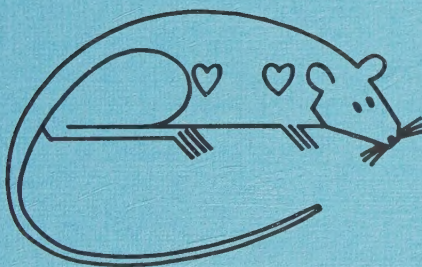


Immunosuppressive Effects Evaluated by the Heterotopic Heart Transplant Model in the Rat

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Malmö 1984

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"... an optimistic mind face the challenge enjoying the spice of difficulty. ... immunity cannot be viewed as a straightforward reaction to a given stimulus. A tidal wave of immunological research has overwhelmed pharmacology and uncovered a multitude of cellular and humoral interactions endowing the immune response with an ever increasing and often bewildering complexity. It is appropriately depicted as a black box where the antigen enters and the immune response emerges. The knowledge of what happens in between is at best fragmentary."

G.L. Floersheim, 1980

Dedicated to the memory of my father

This dissertation is based on the following articles:

- I Konrad P.I. and Husberg B.S.:
The Immunosuppressive Effect of Experimentally Induced Uremia.
Nephron 38:183-187(1984).
- II Konrad P., Forsgren A., Husberg B.S. and Nilsson L.:
The Immunosuppressive Effect of Streptozotocin Induced Diabetes in Rats.
Submitted for publication. Diabetologia, Aug 1984.
- III Konrad P., Husberg B. and Sjögren H.O.:
Enhanced Growth of Colon Carcinoma Isografts in Rats Concurrently Receiving Organ Allografts.
Transplantation Proceedings 8:I(1981).
- IV Konrad P. and Husberg B.:
Prolongation of Heterotopic Rat Heart Allograft Survival by "Methotrexate-Folinic Acid Rescue".
Eur Surg Res 11:331-336(1979).
- V Björk L., Konrad P., Husberg B. and Berggård I.:
Prolonged Survival of Heart Allografts in Rats Treated with Antisera to β_2 Microglobulin.
Transplantation 23:383-385(1977).

CONTENTS

INTRODUCTION	7
AIMS OF THE STUDIES	8
MATERIALS AND METHODS	
Induction and reversal of immunodefficiencies	9
Techniques for the evaluation of immunodeficiency	9
Heterotopic heart transplantation	9
In vitro assay	12
Animals	13
Anesthesia	13
Technique for repeated intravenous drug administration ...	14
Statistical methods	15
REVIEW OF PRESENT INVESTIGATIONS	16
I. The Immunosuppressive Effect of Experimentally Induced Uremia	16
II. The Immunosuppressive effect of Streptozotocin Induced Diabetes in Rats	22
III. Enhanced Growth of Colon Carcinoma Isografts in Rats Concurrently Receiving Organ Allografts	27
IV. Prolonged Survival of Heart Allografts in Rats Treated with Antisera to β_2 -Microglobulin	31
V. Prolongation of Heterotopic Rat Heart Allograft Survival by "Methotrexate-Folinic Acid Rescue"	34
6. The Effect of Methylprednisolone on Heterotopic Heart Transplant Survival	39
GENERAL DISCUSSION	41
CONCLUSIONS	48
ACKNOWLEDGEMENTS	49
REFERENCES	50
Articles I - V and 6	



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INTRODUCTION

The history of experimental transplantation is almost a century old. In 1902 Alexis Carell presented the techniques that are a prerequisite for vascularized organ transplantations and 1905 he and C. Gutrie performed the first experimental heart transplantations in dogs. 1907 a kidney (from a calf) was transplanted to a man for the first time.

It soon became apparent that foreign organs from individuals of the same or a different species are destroyed or "rejected" because of immunological defence mechanisms. The basic physiology of first set and second set rejection was clarified by Sir Peter Medawar (1958).

Whereas immunoreactions are beneficial during infectious conditions and for tumor surveillance they create a major problem after transplantations. Also they can cause auto-immune diseases. Attempts to suppress the immunoreactions mainly by total body irradiation in the 1950:ies resulted in severe side effects. A balance had to be maintained between the desired degree of transplant protection and the risk for serious bone marrow depression and serious infections. Numerous immunosuppressive drugs were tried with variable results (Makinodan et al. 1970). Soon it was also found that certain conditions such as diabetes, uremia and malignant states bring about an unwanted state of immunodeficiency. Few attempts have been made to evaluate these conditions in vivo.

AIMS OF THE STUDIES

When the influence on transplant survival of a specific condition or agent is to be evaluated, a transplant model should preferably be employed. It should allow easy and accurate determination of the graft survival time and should give a rejection course with little variations in control animals. Weak immunosuppressive effects can then be evaluated and a possible in vivo reversibility of the immunodeficiencies can also be detected.

In the studies to be presented a number of agents and conditions with suspected or known immunosuppressive effects of different strength are evaluated by a heterotopic heart transplant model between isogenous strains. The following questions were asked:

- 1) How strong is the immunosuppressive effects caused by chronic uremia? Is the effect reversed after the cessation of the uremic state?
- 2) Does experimental diabetes create a substantial immunodeficiency? Are cellular immunoresponses involved in a deficiency and is it reversed when insulin is administered?
- 3) Can the presence of an allogeneic organ transplant influence the growth of a malignant tumor even if no immunosuppressive treatment is given?
- 4) Can anti β_2 -microglobulin, an antiserum to a component of the major histocompatibility complex on cell surfaces, prolong allograft survival?
- 5) Is methotrexate, a folic acid antagonist, in daily nontoxic doses an effective organ transplant protective agent? Can a toxicity be diminished by intermittent administrations of folic acid?
- 6) How potent are the recorded immunosuppressive effects in comparison to that of a widely used glucocorticoid agent?

MATERIALS AND METHODS

INDUCTION AND REVERSAL OF IMMUNODEFICIENCIES

- I. 30 rats were made uremic by renal resections and contralateral nephrectomies. In 10 of these rats the azotaemia was reversed by renal isotransplantations.
- II. 46 rats were made diabetic by i.v. Streptozotocin (50 mg/kg) injections. In 7 animals the diabetic state was reversed by daily doses of insulin after 7 weeks.
- III. 52 rats received iso- or allogeneic heart transplants in different time relationship to inoculations with DMH-BN 12 tumor cells.
- IV. 10 rats were treated by daily i.v. injections with goat anti-rabbit or rabbit anti-rat β_2 -microglobulins.
- V. 14 rats were given 10 or 25 $\mu\text{g/kg/day}$ of methotrexate. 15 animals were injected with folinic acid 500 $\mu\text{g/kg}$ twice a day in addition to a daily dose of methotrexate (10 or 175 $\mu\text{g/kg}$).
6. 18 rats were given 10-40 mg/kg/day of methylprednisolone in one or two doses.

TECHNIQUES FOR THE EVALUATION OF IMMUNODEFICIENCY

Heterotopic heart transplantation:

A slight modification of the method described by Ono and Lindsay (1969) was used. Donor and recipient animals were anesthetized simultaneously. The abdomen was shaved and the skin cleaned with ethanol solution. Clean but not aseptic conditions were maintained.

After a long midline incision the recipient abdominal aorta and inferior caval vein were exposed by debridement of the retroperitoneal fat. The intestines were packed in saline moistened sponges and retractors were applied. One ml of saline was given intravenously to compensate for expected intraoperative fluid and blood loss.



The donor animals were given 500 IU of heparin intravenously after that a midline incision had been made. The anterior part of the chest wall was transected thus exposing the heart and the major vessels. The superior and inferior caval veins were isolated and divided between silk sutures. The ascending aorta and pulmonary artery were cut 3-4 mm from their origins. A mass ligature was placed around the pulmonary veins. The graft was removed and placed in chilled saline.

In the recipient animal the infrarenal abdominal aorta together with the caval vein was occluded by a rubber coated curved clamp. An operating microscope was positioned and a longitudinal incision was made in the anterior aortic wall. The donor heart was placed between two moist sponges and an end-to-side 10-0 ethilone anastomosis was carried out aorta to aorta. A similar anastomosis was created between the graft pulmonary artery and host caval vein. Small pieces of Spongostan^R were gently pressed towards the suture lines and the clamp was released. The blood loss was usually less than 1 cc and ischemia times were 25-30 minutes.

The abdominal wall was closed in two layers with running Dexon^R sutures and the animals were given 3 cc of saline subcutaneously. They were kept in individual cages postoperatively.

After revascularization the heart transplants rapidly became distended and regained normal colour. Myocardial fibrillations appeared within minutes. They were followed first by multifocal myocardial contractions and then by regular ventricular beats.

The transplanted hearts could easily be palpated in nonanesthetized animals. The intensity of contractions were registered daily by the same person. When oedema and enlargement of the graft were noted two palpations were performed daily. The functional survival times (FST) could thus be determined with 0.5 days accuracy. When regular heart beats could no longer be palpated an exploration of the graft with the rat in ether anesthesia was carried out. If the

absence of contractile beats was then confirmed, the FST of the graft was considered to be ended. In less than 1% of all experiments an adjustment with 0.5 days of the previously estimated rejection time was made after the exploration.

Comment

The heterotopic transplanted heart is perfused by arterial blood through its coronary vessels. The blood returns to the recipient animal by the pulmonary artery after having passed the coronary sinus, right atrium and right ventricle (fig 1). Thus, although the left atrium and ventricle are empty, the blood circulation in the whole heart muscle is maintained resulting in regular muscular contractions. Approximately 5% of the recipient cardiac output passes through the heterotopic heart transplant (Abbott et al. 1964). The technical failure rate (cessation of transplant function or death of the recipient rat within three days after transplantation) was below 5% in control animals and all test groups where untreated rats were subjected to the procedure. However, a considerably higher mortality (10-30%) occurred in uremic and diabetic animals. No paraplegia was observed after the aortic cross clamping.

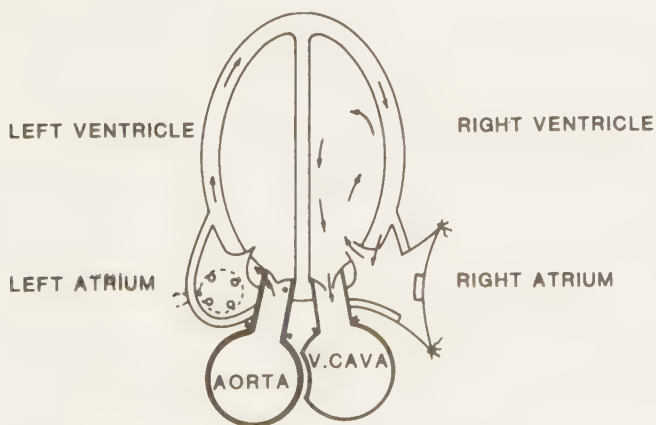


Fig 1 Blood circulation in the heterotopically transplanted heart.

FST is defined by Barker and Billingham, 1971, as "the time at which pulsations became so feeble that it seemed unlikely that it could have maintained the circulation". As this definition appeared somewhat vague a modification more easy to standardize was used in our experiments as described above.

Whereas the exact determination of the graft rejection in non-immunosuppressed recipients is seldom problematic, doubts can arise during a prolonged modified rejection course. More sophisticated methods for the determination of heart transplant survival by ECG registration has been described. Such ECG recordings necessitates repeated anesthetics in each experimental animal. Also, variable positions of the electrodes can cause difficulties in the interpretations (Lee et al. 1970). Moreover, there is no general agreement about the ECG signs of rejection (Abbott et al. 1965, Lee et al. 1970, Heron 1972, Groenewald and Kort 1981). A number of authors regard total loss of ECG activities the end point of the rejection process (v.Bekkum et al. 1969, Freeman and Steinmüller 1969, Boyd et al. 1981). The absence of electrical activity in a transplanted heart is more likely to correspond to "total survival time" than to the often preferred "functional survival time". In the present study the simple and atraumatic palpation and if necessary inspection of the heart transplant was found to be a reliable and reproducible method for the determination of rejection time. Also repeated ECG recordings during anesthesia are difficult to organize in large series of rats and would be hazardous to carry out in sick animals. Recently an ingenious but relatively expensive method for continuous ECG registration with an implanted transmitter has been described (Groenewald and Kort 1981). Again this technically complicated method is difficult to use in large numbers of animals.

In vitro assay

The phytohemagglutinin (PHA) assay as described by Bøyum (1968)

was applied in study II. The animals were immunized by sheep red blood cell suspensions 5 days prior to splenectomy. Lymphocytes were isolated from the devided spleens and cultured. After incubation with PHA ^3H thimidine was added and the incorporated radioactivity was measured. The median number of counts per minute from each triplicate set was used for statistical calculations.

ANIMALS

Brown Norway (BN) isogenous rats of both sexes were used as transplant recipients. In the early studies equisexed Wistar Furth (WF) rats were heart donors. Later they were replaced by (BN x WF) F_1 hybrids. All animals were given water and pellets ad libitum.

Comment

F_1 hybrid rats donated the hearts in the later studies as a test system with longer unmodified transplant survival times and no possible graft versus host element was then obtained. Such a system is more suitable for studies of weak immunosuppressive effects. Both the BN and WF strains were originally obtained from Prof S O Sjögren, the Wallenberg Laboratory, Lund, and were maintained by strict sibling mating. At least twice a year heart transplantations within the strains were carried out resulting in transplant survival times in excess of six months. WF rats were not used as transplant recipients as shorter graft survival times were then obtained (Petirossi et al. 1976 and own observation).

ANESTHESIA

In the earlier experiments pentobarbital (40 mg/kg) was given intraperitoneally before the transplant procedures. In the later studies chloralhydrate (360 mg/kg intraperitoneally) was used. During very short anesthetics or when surgery was performed on weak animals ether was used in a gauze filled plastic tube placed over the nose of the rats.

Comment

Chloralhydrate creating a shorter anesthesia than pentobarbital was found more useful during organ transplantation. Anesthesia can easily be prolonged by administration of drops of chloralhydrate solution on the peritoneal membrane, if necessary. During ether anesthesia resucitation was easily achieved by "mouth to nose breathing" through a plastic tube in case of respiratory depression.

TECHNIQUE FOR REPEATED INTRAVENOUS DRUG ADMINISTRATION

A permanent catheter was designed to facilitate repeated administrations of drugs to nonanesthetized animals. A Venflon^R wing equipped plastic catheter was fitted with a thick rubber membrane in its sidehole. The catheter was extended with a tapered PE 50 polyethylene tube. The device was placed subcutaneously, the side hole being allowed to protude through the interscapular skin. The tapered tip of the catheter was passed into the inferior caval vein through a femoral venotomy (fig 2). Following every injection the catheter was rinsed with 0.3 ml saline. At the termination of an experiment the position and patency of the catheter tip was always checked.



Fig 2 Device for repeated intravenous injections.

STATISTICAL METHODS:

The significance of recorded differences was evaluated by Student's t-test when homogenous distribution of test results were present and by Mann-Whitney (Rank-sum) test when not.

REVIEW OF PRESENT INVESTIGATIONS

I. THE IMMUNOSUPPRESSIVE EFFECT OF EXPERIMENTALLY INDUCED UREMIA

Background:

Already at the time when the first human kidneys were transplanted, interest was focused on a possible altered immune competence of uremic patients (Hume et al. 1955). Lawrence (1966) even described uremia as "nature's immunosuppressive device". Also an increased susceptibility to infectious diseases was reported in uremic patients (Balch et al. 1955, Merrill 1968, Montgomerie et al. 1972).

The immunosuppressive effect caused by uremia is difficult to evaluate in man due to the existence of several background factors that are difficult to standardize. Differences exist in etiology of the uremic state, nutritional status, medications, age and duration of uremia. Dialysis treatment or reversal of uremia by kidney transplantation has been assumed to normalize the possibly deficient immunocompetence in uremic patients, but so far the clinical and in vitro data in support of this suggestion are contradictory (Dobbelstein 1966, Touraine et al. 1975, Birkeland 1976, Quadracci et al. 1976). The altered immunocompetence in uremic animals is extensively discussed in the literature but the results from different studies are inconsistent. Although evidence for an immunosuppressive effect is presented in a majority of publications some authors have found normal immunoresponses (page I:6, Friedman et al. 1980).

Our experiments were designed to produce a stable, standardized non lethal uremia and to examine its possible immunological defect by an organ transplant model. The availability of an isogenous strain made a reversal of the azotemic state by renal is transplantation possible.

Induction of experimental uremia:

Experimental uremia was produced by a modification of the method described by Platt et al. 1952. The rat was anesthetized with ether and a laparotomy performed through left paramedian incision. The left kidney was dissected free from the perirenal fat and adrenal gland. The renal vessels were temporary clamped and the kidney was immobilized with pins on a plastic mould to achieve a standardized resection. Special care was taken to avoid damage of the renal pelvis. After excision of the renal poles and lateral surfaces approximately 15-20 % of the renal mass remained (Ormrod and Miller 1980). Blood loss was minimized by the application of Spongostan^R by manual compression on the cut surfaces after the release of the vessel clamp. When hemostasis had been achieved localized irradiation of both the anterior and posterior side of the kidney remnant was performed in order to prevent regeneration. A "Dermobile" X-ray generator was used, operated at 45 kw, 28 mA for 0.3 minutes with a 0.5 mm filter placed close to the kidney. 290 rad was given totally (Sterner 1979). The intestines and spleen were protected by lead shielding. One week later the right kidney was removed under ether anesthesia.

Comment

Experimental uremia of varying duration can be produced in several ways. Injections of nephrotoxic substances (Nayman 1964), ligation of the ureters (Kroe and Vazquez 1967) or branches of the renal arteries (Lee et al. 1964) have been advocated. The subtotal renal resection and contralateral nephrectomy used in the present experiments result in a stable, moderately severe uremia in approximately 80 % of the animals. The nephrectomy has to be performed separately as an one-step procedure is accompanied by a considerable mortality. Absence of normal weight gain, albuminuria, hypertension and cardiac hypertrophy can be demonstrated in rats that were made azotemic by renal resections (Chanutin and

Ferris 1932). A constant, at least six fold, rise in serum creatinine and elevated serum potassium levels have been observed by us and others (Sterner 1979).

Isogenic renal transplantation.

The method described by Lee 1967 was modified. In the donor animal the right kidney was dissected free through a midline incision and 500 iu of heparin was injected intravenously. The aorta was ligated and divided distal to the origin of the renal artery. The kidney was removed with an aortic segment, a caval patch and one cm of ureter. End-to-side anastomoses were performed in a way similar to that described for the heterotopic heart transplantations (page 9). The kidney graft was placed in a plastic cup and was continuously irrigated by chilled saline during the procedure (Harvig and Norlén 1980). After revascularization an end-to-end uretero-uretero anastomosis was performed with interrupted 10-0 ethilon sutures over an indwelling polyethylene catheter (PE 10) as described by Frödin and Engberg 1975. The subsequent heart transplants were placed distal to the kidney grafts.

Results:

Allogeneic heart transplant survival was found to be 11.6 ± 1.4 days after three weeks of uremia, while controls and sham operated rats rejected their transplants after 8.6 ± 0.4 and 8.4 ± 0.4 days respectively (fig 3). Longer uremia duration did not result in more prolonged graft survival. Reversal of the azotemic state by kidney isografting after 12 weeks and subsequent heart transplantations resulted in rejection times identical with those seen in the control groups.

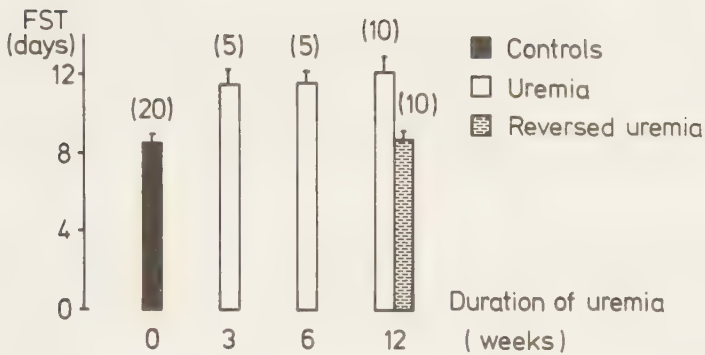


Fig 3 Functional survival time (FST) of heart allografts in uremic rats. Mean + SD (n).

Comment

The results of our experiments are in agreement with those of skin transplantation in rabbits and rats during short- (Smiddy et al. 1961) or longlasting (6 months) uremia (Morrison et al. 1963). Also, similar results have been presented by Souhami 1973 with the use of an organ transplant model in inbred rats that had been uremic for 7-10 days. In a study by van Schilfgaade and van Breda Vriesman (1981) acute, severe uremia in rats was found to prolong the survival of transplanted kidneys markedly. The delay of rejection was thought to be organ specific as heart allograft survival during acute transient azotemia was unchanged.

Immunosuppressive effects from the local irradiations of kidney remnants or a catabolic state of the graft recipient rats are not likely to contribute significantly to the immunodeficiency.

No prolongation in graft survival was seen among non-uremic irradiated control animals. Also the immunosuppressive effect was reversed so soon after the termination of uremia that it is not likely that a recovery from a severe catabolism could have taken place.

The mechanisms behind the immunosuppression in uremia are not clear in spite of numerous studies (page I:6). The results of in vitro tests both in experimental animals and humans are disparate. Humoral factors have been emphasized as being at least partly responsible. Raska et al. (1980) isolated a macromolecular very low density lipoprotein from uremic rat serum that could inhibit mixed lymphocyte reactions in the same species. The in vitro reactivity of human uremic lymphocytes to mitogens was unaltered in the presence of normal serum, but when uremic serum was added to lymphocyte cultures from both normal and uremic patients the response was depressed (Touraine et al. 1975). Newberry and Sandford (1973) claimed a non-dialyzable factor from uremic serum to be responsible for the depression in phytohemagglutinin reactivity of human lymphocytes. These observations suggest that an inhibitory serum factor is present in chronic renal failure. Several such inhibitors have been described. Nelson and Penrose (1973) identified a non dialyzable macromolecular factor which inhibited the DNA syntetic response of normal lymphocytes when phytohemagglutinin was administered.

Recently the role of "middle size" substances (molecular weight 500 - 5000) have been emphasized (Navarro et al. 1982, Mabucci and Nakahashi 1983). When "middle size" fractions isolated from uremic patients were administered to rats, skin allograft rejections were delayed (Navarro et al. 1977). Hanicki et al. (1976) isolated an inhibitory factor from the dialysate of uremic patients (molecular weight below 500) which caused an inhibition of normal human lymphocyte transformation and leucocyte migration. Also elevated

levels of more simple chemical compounds, as methylguanidin, that are present in uremic serum, can impair in vitro functions of normal human lymphocytes (Harris et al. 1972). The classical "uremic" product urea does not inhibit such functions (Silk 1967).

The immunodeficiency caused by uremia could be enhanced by a simultaneously present pharmacological immunosuppression. A synergistic effect was found when azothiaprine (an agent with questionable effectiveness in rodents) was given to uremic rats (Souhami et al. 1973). In contrast chronic uremia in rats did not enhance the immunosuppressive effect of cyclophosphamide, methotrexate or azothiaprine, in vivo (Miller et al. 1982). Häyry et al. (1982) considered the immunodeficiency of uremia to be weak in comparison to that caused by administered immunosuppressive drugs. This view was based on results from fine needle biopsies of rejecting kidneys in patients with a high or low degree of uremia. On the other hand uremia have been found to convey a strong depression of the number of blood lymphocytes and B cell responses to mitogens in humans (Quadracci et al. 1976).

To our knowledge the effect on in vivo immune competence by uremia reversal has not been studied previously in experimental animals. From our experiments one could presume that a relatively weak immunosuppressive effect in an uremic patient vanishes rapidly when a transplanted kidney resumes its function.

II. THE IMMUNOSUPPRESSIVE EFFECT OF STREPTOZOTOCIN INDUCED DIABETES IN RATS.

Background:

Patients suffering from insulin dependent diabetes mellitus have a high susceptibility to infectious diseases (Johnson 1970). An immunodeficiency has been suspected and also verified in several clinical studies on insulin treated diabetic patients (Buschard et al. 1980). However, the exact mechanism behind this defect remains unknown. Experimental studies have not given definite proofs whether cellular or antibody mediated immunological sequences are primarily affected, (for references see page II:11) nor is the influence of insulin on the immunodeficiency clarified (Nichols et al. 1978). In the few transplant experiments that have been performed in diabetic animals skin transplants rather than organ grafts have been utilized. The purpose of the present study was to detect a possible immunodeficiency of experimental diabetes in the rat and to analyze the etiology by insulin treatment and in vitro assays.

Results

Experimental diabetes was induced by streptozotocin (STZ) injections. A markedly prolonged graft survival, 18.1 ± 4.8 days, could be demonstrated after four weeks of such diabetes (fig 4). No further prolongations in graft survival occurred after 8 and 12 weeks. In control animals FST was 8.6 ± 0.4 days. Slightly elevated serum creatinine level were found in the 12 week group. When heart transplantations were carried out after 7 weeks of unmodified diabetes and 1 week of insulin treatment, graft rejection times identical to those in control animals were obtained. The spleens from a number of diabetic rats were harvested and used in a phytohemagglutinin stimulation assay. A decreased mitosis activity as measured by the amount of incorporated ^3H thymidin was noted in rats that had been diabetic for 12 weeks (page II:9).

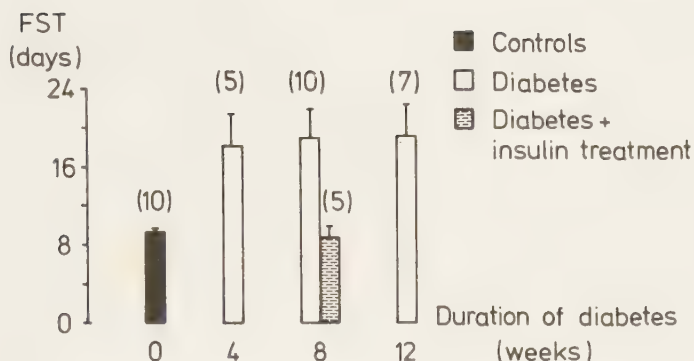


Fig 4 Functional survival time (FST) of heart allografts in diabetic rats. Mean + SD (n).

Comment

STZ was chosen as diabetogenic agent in the present studies. This glucosamin-nitrosurea compound with antibiotic properties has become a widely used agent to produce experimental diabetes. It has more or less replaced the traditional compound alloxan. Its diabetogenic properties was first described by Rakietyen et al. (1963). Mice and rats are very sensitive to the drug and diabetes can be induced in a reproducible way. Man is resistant to the diabetogenic effect of STZ and clinically the drug is used in the treatment of islet cell adenomas, carcinoid tumors and Hodgkins disease (Weiss 1982). The mode of action of STZ in animals is by reduction of nicotinamide adenine dinucleotide in pancreatic cells.

Insulinitis and selective irreversible degeneration of the insulin producing β -cells follow (Weiss 1982). Symptoms much like those of human diabetes as hyperglycaemia, polydipsia, polyuria, glycosuria, cataracts and weight loss occur. However, the finding of weight loss is not constantly observed in the literature (Rakieten et al. 1963, Tancrede et al. 1983). Nicotinamide when injected a few hours after STZ can prevent the diabetes (Rakieten et al. 1971). It is documented that several factors play a role in STZ diabetes induction. Rossini et al. (1978) and Hahn (1983) showed that mice can be protected from STZ effects by immunosuppressive (ALS) treatment or irradiation. Paik et al. (1980) found that thymus dependent lymphocytes play an important role in the development of diabetes in mice while Leiter et al. (1983) claimed that T lymphocytes are not necessary for the diabetes induction by STZ when the drug is given in multiple low doses. As irradiation of mice prior to STZ injections can prevent the induction of diabetes it must be presumed that radiosensitive lymphoid cells participate etiologically.

As mentioned above alloxan administration was previously the standard method to create diabetes in experimental animals. Several differences in the diabetic status as compared to that of the STZ model are documented. Although the hyperglycemia and insulin sensitivity are similar in both kinds of diabetes, plasma free fatty acids and blood ketons are not elevated in STZ treated animals (Mansford and Opie 1968). Alloxan has a higher nephrotoxicity, its action is less specific to the β cells and there is also a narrow margin between general toxicity and diabetogenic effect. Thus a higher mortality has to be anticipated (Junod et al. 1967). The diabetic state in STZ treated mice is irreversible while remissions of alloxan diabetes occur at times (Rerup and Tarding 1969). Impaired antibody responses have been found by some but not all authors (Dolkart et al. 1971, Pavelic et al. 1978). Finally a moderate prolongation in skin transplant survival, not

completely reversible by insulin treatment, has been described in alloxan diabetic mice (Pavelic et al. 1978).

In the experiments presented here a duration of diabetes not exceeding 12 weeks was chosen to avoid possible influence from a concomittant uremia. Glomerular changes has been described in rats duing long lasting STZ diabetes. However disturbances in kidney function have not been found (Weil et al. 1975). It would be desirable to transplant the diabetic rats immediately after the beginning of insulin therapy. However, one week was needed to find suitable insulin doses as hypoglycemia secondary to too high insulin doses was associated with a high mortality. The number of lymphocytes in peripheral blood and body weights that were depressed in diabetic animals rapidly normalized during insulin treatment. At autopsy the spleens and thymuses of the diabetic rats were found to be diminished in size as described by others (Brown et al. 1977). The 20 % incidence of renal tumors reported by Bleasel and Young (1981) in rats with long lasting STZ diabetes could not be verified. Female rats have been reported to be more sensitive to STZ than male animals (Slijepcevic et al. 1975). Rats of both sexes were used in the present experiments and a noted variance in the tolerance to STZ might, retrospectively, be explained by this fact.

The immunodeficiency state that exists in untreated experimental diabetes is probably not caused by hyperglycemia alone as insulin doses, only slightly corrective of hyperglycemia have been shown to normalize second set skin graft survival (Friedman and Beyer 1977). A catabolic state could contribute to the observed immunosuppressive effects in diabetic rats. Our animals were obviously marked by their illness and were loosing weight although serum albumin, a rather unsensitive parameter, was unchanged at the time when insulin treatment was instituted. The main argument against the importance of catabolism as a major cause of immunosuppressive

effects is the fact that, although weight loss was progressive and even serum albumin levels were decreased after the longest duration of diabetes that was studied, graft rejection times were then almost identical to those found a shorter time after STZ injections. Also, when allogeneic heart transplantations were performed as soon as possible (one week) after that the appropriate insulin dose for a single rat had been established the immunosuppressed state had vanished. It is less likely that an immunodeficiency, had it been entirely caused by catabolism would reverse so quickly.

Evaluation of a possible depression in cellmediated immunoreponse was performed with a PHA stimulation assay. A decreased response by spleen cells from diabetic rats could be demonstrated. This decreased sensitivity to PHA stimulation could not be detected in lymphoid cells from two insulin treated diabetic rats. In our hands the plaque forming assay did not yield reproducible results in spite of the fact that the test simultaneously gave reliable results with lymphoid cells from other rat strains in the same laboratory.

The immunological defects of experimental diabetes mellitus could be created by the lack of insulin alone or by a loss of responsiveness to antigenic stimuli that is inherent in the metabolic defect. Although differences in the pathophysiological mechanisms behind clinical and experimental diabetes exist, knowledge about the capacity of insulin to correct the immunological defects in experimental diabetes is of interest.

III. ENHANCED GROWTH OF COLON CARCINOMA ISOGRAFTS IN RATS CONCURRENTLY RECEIVING ORGAN ALLOGRAFTS

Background:

The common denominator in the presented studies is the effect on heterotopic heart transplant survival by immunosuppressive agents and conditions. Although it might be of theoretical interest to evaluate the influence on transplant survival by a growing malignant tumor, the opposite possible effect has a more obvious clinical importance. Could a latent microscopical cluster of malignant cells be stimulated to grow by the simultaneous presence of a foreign organ transplant?

A vast amount of data largely collected by I Penn (1979) and later by Birkeland (1983) indicate that there is an increased frequency of malignant diseases among transplant patients. The obvious explanation is that a side effect of immunosuppressive therapy is at hand. The risk for a de novo malignancy has been claimed to be increased after treatment courses with several cytostatic and immunosuppressive drugs (Daynes et al. 1979). However, the mere introduction of strong transplant antigens could also facilitate the growth of a latent malignant tumor. The present investigation was designed to evaluate the influence on tumor growth of allogeneic transplants in non-immunosuppressed animals.

Results

52 BN rats were challenged with DMH-BN 12 malignant tumor cells. They received cardiac allo- or isografts 2-4 days before or 2-6 days after the tumor cell inoculations. When allogeneic heart transplantations were carried out 2-4 days before cell challenge a facilitation in tumor growth took place. Identical tumors in isografted BN rats served as controls (Fig 5).

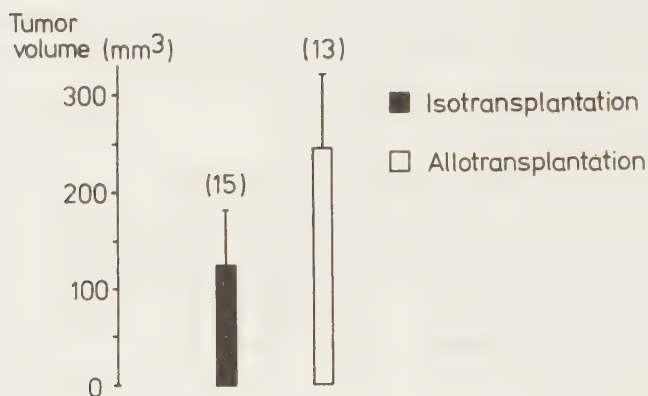


Fig 5 Tumor cell volumes 4 weeks after iso- or allotransplantation. Mean + SD (n).

Comment

It is to be expected that an inhibitory factor will delay the time of onset for rapid growth of an experimental malignant tumor. Once rapid growth has started the tumor should be sensitive only to treatment with mitosis inhibitors until lack of nutrients become a limiting factor. Therefore it is not surprising that the time relationship between tumor cell challenge and heart transplantation turned out to be critical. A clearcut facilitation of tumor growth could be recorded when allogeneic transplantations were carried out during a narrow time interval before progressive tumor growth had started. Nor is it surprising that the differences in tumor volume could only be recorded during a limited time. As simultaneous heart iso-transplantations were performed on control animals challenged with an identical number of tumor cells from the same batch, variations in the experimental conditions

could be ruled out as an explanation for the differences in tumor growth rates.

Instead there are several possible specific explanations. First of all "antigen competition" might be present. This term was first used by Michaelis 1902 who described a phenomenon in which the immune response to one antigen was suppressed by the response to a second unrelated antigen. The early research regarding this finding aimed at a clarification of the practical implications for the use of polyvalent vaccines. Later it became apparent that antigen competition could be demonstrated in a wide variety of immunological reactions. Suppression of Ig-G and Ig-M production (Eidinger et al. 1968), delayed hypersensitivity (Schwarz and Leskowitz 1969), GVH reactions (Miller et al. 1964), transplant rejection (Eidinger et al. 1968) and experimental autoimmune lesions (Einstein et al. 1968) could be noted. Also the sequential administration of two unrelated antigens can lead to enhancement of the immunoresponse to the second antigen or to antigenic competition depending upon which doses and time interval that is chosen for the two antigen administrations (Waterstone 1970). Antigenic competition is best evoked when the first antigen is thymus dependent (Möller 1971). More recent immunological studies have revealed several other mechanisms that could be at work in our experiments:

1. Circulating immune complexes can be created during the course of an allograft rejection (Jordan et al. 1982, Fukuda 1983). Such complexes could affect the tumor growth by blocking the tumor defence factors that are dependent of Fc receptors for their efficiency, i.e. macrophages and K-cells.
2. Prostaglandins can be produced by the rejecting allograft (Tannenbaum et al. 1984). Prostaglandins of the E series have been shown to be inhibitors of cellmediated immunoreactions (Mertin et al. 1984, Staszak and Goodwin 1980).
3. Histamine is often produced during immunoreactions and also during allograft rejections (Dy 1982). It suppresses T-cell re-

sponses (Garovoy et al. 1983) and cellular in vitro assays (Wang and Zweiman 1978). The histamine type 2 receptor antagonist, cimetidine, can augment in vitro proliferative responses by lymphoid cells in rodent and man (Plaut et al. 1973, Gifford et al. 1980).

4. Multispecific suppressor cells can be generated during a MLC reaction (Pazdevska et al. 1983). Such reactions could be considered as in vitro equivalents of rejection processes.

5. A chronic "irritative" agent for the immunoresponse system, e.g. BCG, can enhance the activity of macrophagelike suppressor cells (Davies 1982). It can be speculated that an allograft also creates a long term chronic "irritation" of the immunological defence system.

Clinically, a patient with an organ transplant and a malignant tumor also receives immunosuppressive treatment. The tumor sizes in animals receiving heart allo- or isografts, while being submitted to cyclophosphamide treatment, were therefore compared in a pilot study. The tumor growth rates were depressed to a variable degree and no significant differences in tumor volumes could be found (page III:3).

Thus cyclophosphamide counteracted the facilitating effect on tumor growth caused by the allogeneic transplants.

IV. PROLONGED SURVIVAL OF HEART ALLOGRAFTS IN RATS TREATED WITH ANTISERA TO β_2 MICROGLOBULIN

Background:

β_2 -microglobulin (β_2m) is a protein of low molecular weight (11.800) consisting of a single polypeptide chain. The molecule was originally isolated from urine but is also present in other body fluids (Berggård and Bearn 1968). Structurally β_2m is closely related to immunoglobulin domains (Peterson et al. 1972, Smithies and Paulik 1972). It is present on the surface of almost all mammalian cells, and the protein represents the light chain of major histocompatibility class I antigens (HLA-A, -B and -C antigens in man) (Peterson et al. 1974, Nakamuro et al. 1973). β_2m is also associated with several other components of the major histocompatibility complex (Goodenow et al. 1982). β_2m has been purified from several mammalian species and parts of the molecule are similar in species belonging to different evolutionary stages (Lögdberg et al. 1980, Skalev et al. 1981). β_2m can promote rosetting of sheep red blood cells to macrophages and fix complement. Moreover, β_2m enhances the expression of receptors for the IgG Fc portion on the lymphocyte surface and the molecule might have chemotactic properties. Antibodies against β_2m have several in vitro effects. Thus, both lymphocyte transformation induced by various stimuli, and the action of various cytotoxic effector cells, can be inhibited by anti- β_2m . This suggests a role for β_2m in both the induction and the effector phases of an immunoresponse (for references concerning these in vitro effects of β_2m and anti- β_2m see Berggård et al. 1980). The aim of our study was to examine whether antibodies to β_2m influences the immunoresponse in vivo.

Results:

Rabbit anti-rat or goat anti-rabbit β_2m was given i.v. to transplanted BN rats. Donor hearts were obtained from WF animals. Pooled sera from non-immune rabbits or goats were administered to control rats. Survival times of the transplants are shown in fig 6. The administration of non-immune sera did not result in prolonged graft survival (6.8; 7.0 days respectively) as compared to that in untreated rats. A statistically significant prolongation was achieved by daily i.v. administration of 1 ml (totally 8 ml) goat anti-rabbit or rabbit anti-rat β_2m (8.6 ± 0.8 days). In a separate pilot study five donor hearts were perfused with 1 ml of goat anti- β_2m serum ex vivo before grafting. Another five recipient rats were given daily 1 ml doses of a rabbit antiserum to purified Ag B antigens after the transplantations. No prolongations in graft survival were seen in either of the two groups.

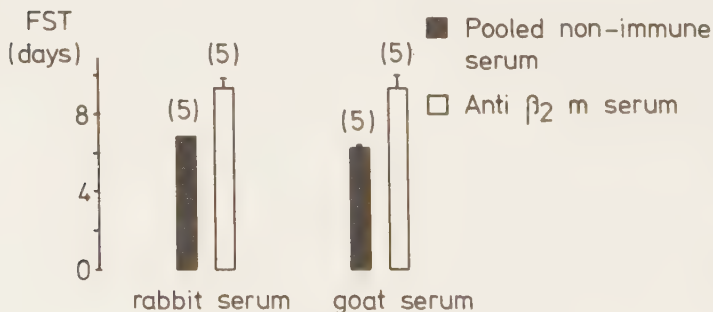


Fig 6 Survival of allogeneic heart transplants (FST) after treatment with anti β_2 microglobulin- or non-immune sera. Mean + SD $^2(n)$.

Comment

From a theoretical point of view, the fact that antibodies against highly purified cell surface proteins are capable of influencing the immune response in vivo, is of interest. This has not been demonstrated previously. In our test system the transplant protective effect was weak. This fact can be ascribed to two factors: The i.v. injected antibodies to β_2m are distributed to the surface of virtually all cells, which means that only a minor part of the antibodies bind to the lymphocytes. Furthermore the BN-WF combination involves strong Ag B differences with short survival times of allografts exchanged between the strains. A stronger graft survival prolonging effect might be achieved in a different rat strain combination. However the purpose of this study was only to confirm in a qualitative way the known in vitro immunosuppressive effects of anti- β_2m in an in vivo system. In the pilot study the donor hearts were perfused by the same anti- β_2m serum prior to grafting in order to achieve a local protection against immunocompetent cells attacking the graft. Under such conditions no prolongation in transplant survival could be found. Also antibodies against purified Ag B antigens administered to the graft recipients in vivo were without effect. These observations suggest that the immunosuppressive effect by anti- β_2m is caused by an interaction with lymphocyte antigens presenting β_2m structures. The effect is not likely to be caused by a simple (and stable) blocking of the classical (Ag B) transplantation antigens.

V. PROLONGATION OF HETEROTOPIC RAT HEART ALLOGRAFT SURVIVAL BY "METHOTREXATE - FOLINIC ACID RESCUE"

Background:

There are mainly three reasons why it is of interest to study the immunosuppressive effects accomplished by the folic acid antagonist amethopterin (methotrexate). Firstly, it has been widely and effectively used after clinical bone marrow transplantation (Storb 1983). Secondly, if severe toxic side effects occur a specific fast acting antidote, folinic acid, is available. The presence of an antidote is unique among the drugs used for immunosuppression and can be life-saving in a critical situation (fig 7). Thirdly, claims have been made that during tumor treatment and after experimental skin transplantations intermittent administration of the antidote can diminish the side effects of methotrexate relatively more than the immunosuppressive effects. This is called the "folinic acid rescue" principle (Sullivan et al. 1959, Makinodan et al. 1970).

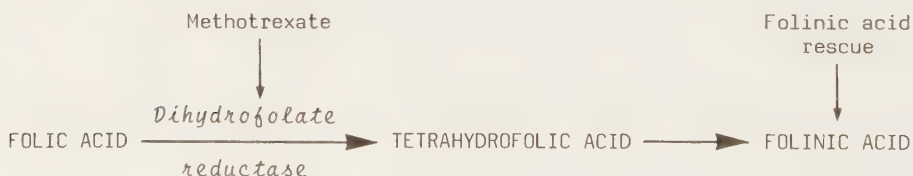


Fig 7 Folic acid metabolism.

The immunosuppressive effect of folic acid antagonists was suggested by Little et al. 1950, who noted lower antibody titers to gram negative enteric bacteria when feeding chicken a diet

deficient in folic acid derivatives from the time of hatching. During the following decade a number of investigations could document a depression in antibody response to test antigens in several mammals such as mice (Malmgren et al. 1952), rabbits (Sterzl 1961), guineapigs (Friedmann et al. 1961) rats (Santos and Owen 1962) and dogs (Thomas et al. 1961). When folic acid antagonists were administered a depressive effect on cellular immune responses was likewise documented (Prichard and Hayes 1961, Turk 1964, Mitchell et al. 1969).

Early experiments with methotrexate alone as immunosuppressive transplant protective agent gave variable results. The survival of skin allografts was found to be substantially prolonged in rats (Santos and Owens 1965), guineapigs (Berenbaum 1963) and dogs (Hechtman et al. 1962). A comparatively weak effect in rats and mice was presented by Kripke et al. (1973) in spite of prolonged treatment with high mortality. Goldin et al. (1962), Glynn et al. (1963) and Floersheim (1964) also found borderline prolongation of skin transplant survival. Varying degrees of protective effect accompanied by severe toxicity were demonstrated in organ transplant experiments (Blumenstock et al. 1963, Hardy et al. 1964, Reemtsma et al. 1962, Zukoski et al. 1962). After experimental bone marrow transplantation in dogs a protective effect against graft versus host reactions at the cost of high toxicity was found (Storb et al. 1970).

The toxicity of methotrexate is similar in humans and rodents, mainly affecting the bone marrow, gastrointestinal mucosa, and to a less degree kidneys and liver (Brodie 1965, Condit et al. 1969, English 1980). Numerous attempts have been made to diminish the toxicity of this powerful immunosuppressive and antiproliferative agent by intermittent administration of the antidote folinic acid. This "Folinic acid rescue" concept has been tried mainly in connection with experimental and clinical tumor therapy (Sullivan et al. 1959, Mead et al. 1963). In animals it has also been used to

prevent antibody responses to test antigens (Berenbaum 1965, Abbott et al. 1971). However, in organ transplant experiments no clear-cut immunosuppressive effect has been demonstrated by methotrexate treatment with or without interposed folinic acid administration (page V:2).

In the design of an organ transplant model for the study of methotrexate-folinic acid effects basic facts concerning drug metabolism, turn over rates, and LD 50 doses for the chosen species must be taken into account. In the literature a vast amount of animal treatment schedules without references to such data can be found. In humans the doses are mostly established empirically (White 1981). Multiple bolus injections or single megadoses of methotrexate with or without folinic acid administration are used in the treatment of leukemia, severe psoriasis and some solid tumors (Salaman 1981).

A considerable interspecies difference exists regarding absorption, excretion and turn over rate of methotrexate, (Henderson et al. 1965, Brodie 1965, Bertino et al. 1967, Zurek et al. 1968, Zaharko and Oliviero 1970). A non-linear plasma clearance curve with a terminal half life of four hours has been described in rats after the administration of three intravenous methotrexate doses (Scheuffler 1981, 1982). The only available mortality data for rats indicate a LD 50 dose of 1,22 mg/kg after drug administration during five days (Santos and Owens 1964). Folinic acid has a short half-life after i.v. administration in rats (Lederle, unpublished data).

Having surveyed the available literature it was clear to us that toxicity studies had to be performed to define the optimal post transplantation long term treatment doses and methotrexate-folinic acid time intervals.

Results:

Daily intravenous doses of methotrexate in amounts of 50 µg/kg

during three weeks caused weight loss and mortality. One daily dose of folinic acid (Leucovorin, Lederle) did not yield significant protection against methotrexate toxicity but when an excess of folinic acid was injected 16 and 20 hours after each methotrexate dose 175 $\mu\text{g/kg}$ and day of the latter drug could be given without mortality. When heart transplantations based on this knowledge were performed a considerable transplant protective effect was noted after daily treatment with 175 $\mu\text{g/kg}$ of methotrexate and an excess of folinic acid (fig 8). However, virtually identical heart transplant survival times were achieved with well tolerable doses of methotrexate alone (25 $\mu\text{g/kg}$ daily).

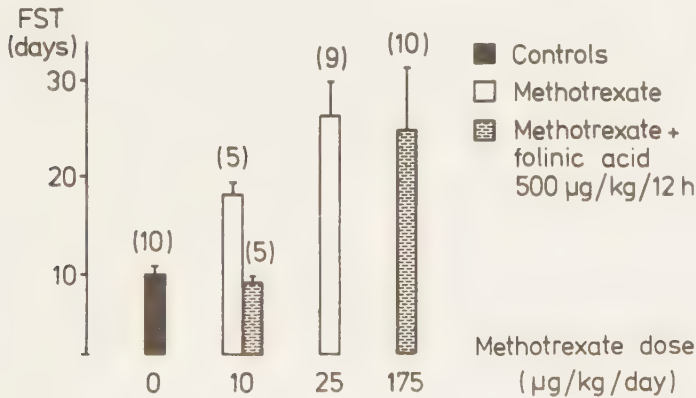


Fig 8 Functional survival time (FST) of allogeneic heart transplants in methotrexate or methotrexate-folinic acid treated rats. Mean $\pm$ SD (n).

Comment

It can be argued that the used Methotrexate dose, 25 $\mu\text{g/kg/day}$, could be less than optimal and that even better transplant survival could possibly be obtained with a daily dose closer to 50

$\mu\text{g/kg}$ without interposed folinic acid injections. However, the purpose of this study was not to define the maximal tolerable methotrexate dose but to see if the "cost-benefit" effectiveness of methotrexate could be improved by folinic acid rescue. Obviously, in the experiments presented here, the hypothesis was not found to be valid. Rather it is a remarkable finding that methotrexate in a daily amount of $10 \mu\text{g/kg}$, that is in a safe and far from toxic dose, can almost double allogeneic heart transplant survival in spite of a strong Ag B histoincompatibility barrier. In the experiments presented here the "rescue" principle was found to be valid only regarding the toxicity. In paper II it is suggested that a different time interval between methotrexate and folinic acid administration could still prove the whole "rescue" principle to be valid (i.e. a decrease in toxicity without a corresponding decrease in immunosuppression). A limited number experiments were performed but neither shortening nor prolongation of the time interval resulted in better graft protection as compared to methotrexate treatment alone.

6. THE EFFECT OF METHYLPREDNISOLONE ON HETEROTOPIC HEART TRANSPLANT SURVIVAL.

Background:

Glucocorticoid agents are known to have a profound effect on transplant survival in all tested species by interference with essentially every part of the immune system (for references see Dupont et al. 1984) The influence of a widely used glucocorticoid agent, methylprednisolone, was tested in the present study. This was done to provide a background to the transplant survival data that were recorded in the studies I, II, IV, V.

Results:

In our test system the prolongation in graft survival caused by intravenous administrations of methylprednisolone once or twice a day was investigated (fig 9). 20 mg/kg, once a day, a high dose according to previous investigations, gave no prolongation in transplant survival. 5 mg/kg of methylprednisolone administered twice a day, that is in a low and safe dose, created a marked prolongation in graft survival (15.0 ± 1.7 days). A four times higher dose, twice a day, gave a considerable prolongation in FST (69.4 ± 11.6) but weight loss and cachexia was noted after months of treatment.

Comment

In rat organ transplant studies the glucocorticoids have usually been administered intraperitoneally (Tinbergen 1968, Shehadeh et al. 1970, Salaman 1983). However, such injections are less suitable in animals that have intraabdominally placed heart transplants. Repeated intramuscular or subcutaneous drug administrations are difficult to standardize due to scarring and infectious risks. Intravenous glucocorticoid administration after allogeneic organ transplantation in the rat has been used to a minor extent and usually in single bolus doses (Bell et al. 1973).

The safe intravenous route that was made available by the permanent catheter (page 14) thus seemed preferable, although scarce data regarding the immunosuppressive and toxic effects caused by long term daily intravenous administration of different glucocorticoid agents are available. It was soon apparent that once a day administrations were not likely to result in transplant protection until subtoxic doses were used.

Steroid side effects and mortality occurred after several weeks of methylprednisolone treatment in a dose of 40 mg/kg/day. The possible long term side effects of smaller steroid doses were never tested as the experiments were terminated when the grafts had rejected.

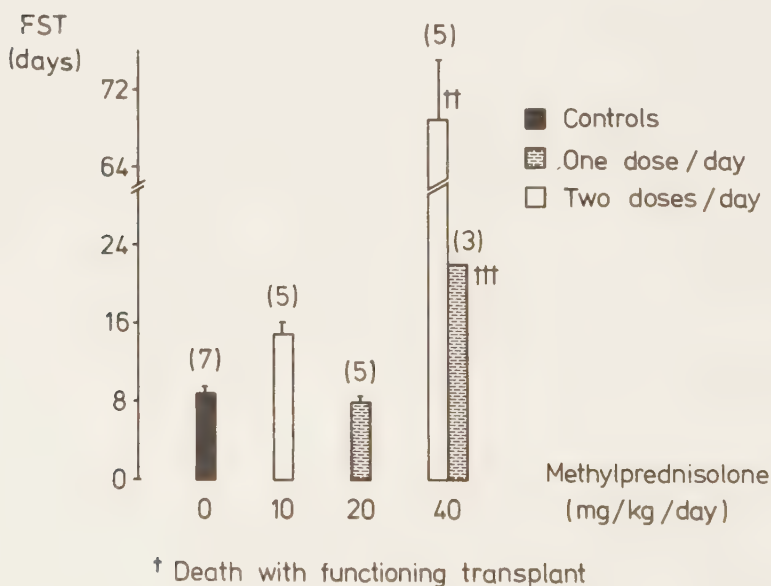


Fig 9 Survival of heterotopic heart transplants during methylprednisolone treatment. Mean + SD (n).

GENERAL DISCUSSION

The purpose of this study was to evaluate several conditions with a definite or possible immunodeficiency. There are numerous in vitro tests available for this purpose ranging from simple determinations of antibody responses to sophisticated recordings of lymphoid cell functions. They are all designed to measure a defined part of the immune response process. It is difficult to evaluate the whole immune response sequence by such methods. The unmodified rejection of a foreign graft requires an immune system that functions normally in all consecutive steps. Therefore to get an overall estimate of the level of immunoresponsiveness a transplant model is preferable.

Several organs and tissues have been transplanted experimentally. Skin transplants are easily and rapidly performed also in small animals and have been used as research tools by many investigators. However, skin in the context of transplant models can not be regarded as an organ. The host is immunized by a lymphatic route and not through the blood stream as when a vascularized transplant is used. Also secondary factors influence the rejection process: Inflammatory reactions at the transplant site can attract macrophages capable of accelerating cellular immune responses. Necrosis of graft tissue can cause the release of antigenic material with adjuvant effects on immunological reactions (Warren et al.1973). Last but not least the determination of rejection times for skin transplants is difficult to standardize as the total necrosis of the graft, which is usually regarded as an end point, develops during several days. Thus, skin transplantation does not constitute a satisfactory transplant test model. Besides the heart, a number of organs such as kidney, pancreas, liver and intestines are used for experimental vascularized transplantation experiments. However, the latter organs are less useful for the evaluation of immunoresponses because of difficulties in the determi-

nation of exact rejection times. For example in a kidney graft experiment the only easily defined end point for rejection is the death of the animal. This can occur several days after the cessation of graft function.

None of these disadvantages burden the cardiac allograft model. On the other hand it is a relatively complicated and technically demanding method. The first systematic study on "Transplantation of the intact mammalian heart" was presented by Mann et al 1933. Abbott et al published a heterotopic model in rats 1965. A further technical development by the use of microvascular technique with end-to-side anastomoses between the heart vessels and the abdominal aorta and caval vein was published by Ono and Lindsay 1969. The method has become a suitable tool for the study of immunological processes and was chosen in the present experiments although with minor technical modifications. A simplified heterotopic heart transplant model was published by Heron 1971 using non-suture anastomoses to the cervical vessels. Shorter operation times and diminished blood loss were claimed to result. The technical failure rate, due to thrombosis of the anastomosed vessels, was reported to be 10 - 15 % (Olausson et al. 1984). This should be compared to the (at most) 5 % obtained with the use of suturing techniques in our series. Also "skin-to-skin" time was regularly less than 60 min in our experiments.

An other advantage of the heterotopic heart transplant model is that the host survives after a complete rejection. Thus further studies of the recipient animals can be done. Moreover no spontaneous tolerance to heart allograft occurs, in contrast to for instance liver and kidney grafts (Houssin et al. 1979; Pettrossi et al. 1976).

In identical donor-recipient combinations skin has been found to be more "rejectable" than heart, heart more rejectable than kidney and kidney more rejectable than liver. Lesser vulnerability of

kidney grafts compared to skin transplants was claimed to be one of the possible reasons for differential graft survival times in rats (Sakai 1969). In a previously cited study (van Schilfegaade and van Breda Vriesman 1981) kidney but not heart graft survival was found to be prolonged in acute uremia.

Surgical procedures are more easy to perform in bigger animals such as sheep, dogs, pigs and cats. However, the rat has become the most common species used in experimental organ transplantation for the following reasons:

1. Inbred "isogenous" strains are available.
2. Rats are big enough as to allow any surgical procedure with the use of microsurgical techniques.
3. The rat has the basic anatomy and physiology of a mammal.
4. Breeding is cheap due to large frequent litters and short growth period.
5. Excellent resistance to infections makes sterile precautions during surgery unnecessary even in immunosuppressed animals.

When experiments are carried out with the use of isogenous strains all variations in the results caused by genetic factors are kept at a minimum. Transplants between members of the same strain survive indefinitely without signs of rejection. When organs are transferred between two isogenous strains graft survival times with small variations can be obtained. In the studies presented here the survival of cardiac grafts in allogeneic control hosts showed a maximum variation of ± 0.5 days within the different studies.

Five histoincompatibility systems, Ag A-E, are known in the rat. They differ in tissue distribution and immunogenicity (Palm 1971, Palm and Wilson 1973). The Ag B system is considered to have the strongest influence on organ graft behaviour. It is regarded as the equivalent of the HLA system in man and the H2 system in mice.

The BN (Ag B3) and WF (Ag B2) strains used in our experiments have different Ag B antigens and thus have a major histoincompatibility. This is reflected by a relatively short heart transplant survival in most test groups. The antigenic differences are naturally less pronounced when a F_1 hybrid animal between the BN and WF strains is used as an organ donor and a prolonged survival (1 - 2 days) is obtained after transplantation.

A certain prolongation in control graft survival times could be observed during the course of years and therefore a contemporary control group was always included when a series of heart transplantations was undertaken in immunosuppressed animals.

In the present experiments a (BN x WF) F_1 hybrid donor to BN recipient combination was used in all studies except one (IV) where WF rats served as donors. Therefore comparisons between the prolongation in graft FST's in study IV versus the studies I, II, V, VI must be made with caution. On the other hand the exact weighing of maximum obtainable prolongations in the different studies is not the aim of this presentation. Such exact comparisons demand preliminary experiments to establish long term LD 50 doses and optimal pretransplant durations of immunodeficiencies. Also, such studies must be performed with the same rat strains that are used in the present experiments and in a scale that is quite unrealistic in a thesis project. Rather, the magnitude of prolongations in FST is determined so that a single immunosuppressive state can be labelled as "weak", "moderate" or "strong" (arbitrary prolongations: < 4 days, 4 - 14 days, and > 14 days respectively).

Major surgical trauma might influence the immunoresponses. Although it has been claimed to cause an immunosuppression in a majority of studies in the literature, ultimate proof still does not exist and thus the effect is questionable. Payne et al 1984 reviewed previous reports on possible immunosuppressive effects on

lymphocyte transformation in humans. They found nine publications in favour and five against such an effect. Differences in experimental conditions and large inherent variations in the PHA stimulation lymphocyte transformation tests were thought to explain the discrepancies. In their own study no differences could be found between pre- and postoperative lymphocyte transformation tests. In studies in mice the amputation of a limb produced an increased suppressor cell activity (Wang et al. 1980, Lundy and Ford 1982). The activated splenic suppressor cells appeared to have phagocytic properties and the suppression could be reversed by prostaglandin inhibitors (Wang et al. 1982).

Kinnaert et al (1980, 1984) found evidence in rats of stimulated antibody responses against sheep red blood cells in proportion to the magnitude of the surgical trauma. They also pointed out that the altered immune reaction can not be detected in in vitro studies. Excessive postoperative stress has been described to prolong both heart and kidney allografts in rats (Kort et al. 1979). In rats rendered uremic by a surgical method and in sham-operated rats a depressed lymphocyte function as compared to control, nonmanipulated animals has also been described by Miller and Stuart 1980.

In our studies a possible influence on the results by surgical trauma can generally be ruled out as the control animals underwent the same surgical procedures as the rats belonging to an experimental group. In study II the control rats were even subjected to isogenic heart transplantations to create identical trauma effects. However, in study IV this ambition could not be carried through completely. It was not possible to give the control non-uremic rats a surgical trauma identical to that created by the renal resections. Also they were not isografted to "sham reverse" an uremia although they were irradiated and underwent rightsided nephrectomies. However this would have created a problem in the evaluation of the reversibility of the immuno-

deficiency only if the graft FSTs would not have normalized completely.

Several agents and conditions with known or suspected immunosuppressive effects were evaluated in the test model now outlined.

Chronic uremia had a weak influence on the immunoresponsiveness. A number of factors such as catabolism, hyponutrition and toxic substances might be involved. It has been difficult to evaluate the relative importance and strength of these factors. However, the kidney isotransplantations performed in our experiments reverse the immunodeficiency and it is possible to draw conclusions from the rapidity of the recovery. The fast restitution of immunocompetence makes the elimination of a serum factor likely.

A moderate transplant protective effect was seen in severe diabetes. The immunodeficiency is directly or indirectly caused by a lack of insulin as this hormone can reverse the defect. Although in vitro immunoassays are problematic in BN rats, it is clear that cellmediated immunoresponses are defective in streptozotocin diabetic rats. A very low density lipoprotein has been found to be an inhibitor of lymphocyte responses in both uremia and diabetes in rats (Raska et al. 1980, Chi et al. 1982). Thus common mechanisms can be present to some extent in the two conditions.

No significant prolongation of transplant survival was recorded in the presence of a malignant tumor. However, the opposite effect, that an allogeneic transplant facilitates the growth of a simultaneously present malignant tumor, is of great interest, as human organ transplant patients could have latent tumors.

Weak transplant protection could be noted when antisera to β_2 -microglobulin were administered. Such an in vivo immunosuppression has not previously been demonstrated. The mechanism behind the prolongation in graft survival remain unclear.

Methotrexate was found to have a strong immunosuppressive effect in low non toxic doses. When folic acid was administered intermittently higher methotrexate doses could be given without signs

of toxicity. However, this did not result in any further improvements of efficacy for transplant protection.

Methylprednisolone was the most potent immunosuppressive agent tested. This is in agreement with the clinical and experimental experience of many authors. The steroid effect is comparable to that found during treatment with the new drug Cyclosporin A as judged from organ transplant experiments by Jamieson et al. 1979 in a strain combination similar to ours.

In human transplant practice two or more immunosuppressive agents are usually applied simultaneously. In addition the different underlying diseases can cause immunodeficiencies. Therefore it is difficult to evaluate the immunodeficiency caused by a single factor in clinical studies. Such studies require an animal model with small variations in rejection times caused by other factors than the one, that is being studied. Also, the end point of rejection should be easy to determine accurately and the process should not effect the general condition of the animal.

The answer to the wishes is the heterotopic heart transplant model in isogenous rat strains.

CONCLUSIONS

From studies on in vivo immunocompetence using the heterotopic heart transplant model the following conclusions can be drawn.

1. Experimental chronic uremia causes a weak immunosuppression. The effect vanishes quickly and completely when the azotemic state is reversed.
2. Untreated streptozotocin-induced diabetes gives an immunodeficiency of moderate strength. It can be reversed rapidly by insulin treatment. Defective cellbound immunoresponsiveness is involved in the deficiency.
3. A heart allograft facilitates the growth of a concomitant malignant tumor.
4. Antisera to β_2 -microglobulin produce a weak immunosuppressive effect in vivo.
5. Methotrexate in low, non-toxic doses creates a strong transplant protection. Intermittent administration of folinic acid cannot change the relationship between immunosuppressive effect and toxicity for methotrexate.
6. Methylprednisolone has the strongest immunosuppressive effect among the tested agents and conditions.

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The Immunosuppressive Effect of Experimentally Induced Uremia

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Key Words. Experimental uremia. Immunosuppression. Heart transplantation. Rat.

Abstract. Isogenic BN rats were made uremic by subtotal renal resections. After different periods of uremia the possibility of an immunodeficiency was evaluated by (WF x BN) F₁ BN heterotopic heart transplantations. A prolongation of transplant survival was found that was not related to the duration of uremia. In some rats the uremia was reversed by isogeneic BN BN kidney transplantation before cardiac grafting. An immediate reversal of the immunodeficiency occurred. Thus an immunosuppressive effect by uremia can be documented, and to some extent quantitated, by heterotopic heart transplantation between rats of different isogeneic strains.

Considerable efforts have been made to evaluate the apparent immune deficiency of the azotemic state during the last decades. Observations in humans are complicated, as variances in causative disorders can influence the applied immunological tests. Differences in patient age, medications and dialysis treatment add to the difficulties to achieve homogeneous test conditions (12). In vitro tests, both in humans and in experimental animals, naturally can only give knowledge of isolated parts of the immune response

sequence. They are also influenced by numerous laboratory test factors (26, 34). An animal organ transplantation model can give information regarding the overall influence on the immunological functions by an azotemic state. Such studies have so far been hampered by large biological differences between the test animals. By use of inbred rats with stable, surgically induced uremia and an organ transplantation model, standardized test conditions for an in vivo evaluation of possible immune deficits are obtained.

Materials and Methods

General Outline of the Experiments.

Uremia was induced by subtotal renal resections in isogenic rats. After different time intervals heterotopic heart transplantations were performed with the use of donors of an Ag-B-different isogenic strain. Graft survival time was recorded. In one group of animals the uremic state was reversed by isogenic kidney grafting before allogeneic heart transplantation.

Experimental Groups

Group I. 20 rats received allogeneic heart transplants 3, 6 and 12 weeks after induction of uremia.

Group II. In 10 rats, the uremic state was reversed by isogenic kidney transplantation 12 weeks after uremia induction. 1 week later, allogeneic heart transplantations were performed.

Group III (Controls). (a) 10 normal rats received allogeneic heart transplants; (b) 10 sham-operated animals (left kidney irradiated but not resected, right kidney removed 1 week later) received heart allografts 3 weeks after the right-sided nephrectomies. (c) 10 normal rats underwent isogenic heart transplantations.

Animals

Isogenic male and female BN rats (175-340 g) were used for uremia induction. Rats of the same strain were also donors for isogenic kidney transplantations. (BN x WF) F_1 rats of 200-300 g served

as donors for heterotopic allogeneic heart transplantations. All animals were fed on pellets and water ad libitum.

Surgical Techniques

Uremia Induction. The left kidney was dissected free under ether anesthesia and resection of the renal poles and cortical surfaces was carried out with removal of approximately 75% of the renal mass (24). Bleeding was controlled by temporary clamping of the renal pedicle and manual compression of the resected surfaces. When hemostasis had been obtained, localized irradiation of the kidney remnant was administered (290 rad) to prevent regeneration (33). Spleen and intestines were protected by lead shielding. 1 week later, a right nephrectomy was performed under ether anesthesia.

Heart transplantations. The procedures were performed under chloral-hydrate anesthesia using the microsurgical technique described by Ono and Lindsay (25). The cold ischemic periods were shorter than 30 min. The technical failure rate (postoperative mortality or nonbeating transplants after 3 days) was below 5% (10% in the 'reversed uremia' group). The functional survival time (FST)(2) was registered by palpation of the transplant in unanesthetized animals twice a day, thus allowing the determination of rejection time with 0.5 day's accuracy. When cardiac arrest was suspected, an exploration of the graft was carried out to ascertain FST by macroscopical examination. In 2 cases out of 60, a prolongation of the previously estimated rejection time with 0.5 days was done after the explorations.

Renal Transplantations. Isogeneic kidney transplantations were performed under chloral hydrate anesthesia using sex-matched BN donors. A modification of the technique by Lee (14) with use of intraoperative cooling of the kidney graft and a uretero-uretero anastomosis was applied. The technical failure rate (postoperative

mortality or serum creatinine level $>110 \mu\text{mol/l}$ (1.2 mg/dl) after 1 week) was 16%. In the reversed uremia group the subsequent heart transplant was placed distal to the kidney graft.

Definition of Uremia

Uremic state was defined as an at least fourfold rise of serum creatinine level as compared to preoperative values. Animals with serum creatinine of $250\text{--}400 \mu\text{mol/l}$ ($2.7 - 4.4 \text{ mg/dl}$) 1 week after right sided-nephrectomy entered the study. The level of uremia in these rats then remained constant. Highly uremic animals usually progressed in their azotemic state and were either too sick for further surgical procedures or died within 3-4 weeks. The uremic state was monitored by repeated registration of body weight, WBC and lymphocytes as well as creatinine and albumin levels in serum.

Laboratory Tests

Blood samples were obtained from a tail vein of unanesthetized animals. Routine laboratory technique of the hospital were used.

Statistical Methods

$p < 0.01$ in Student's t test or Rank sum test was considered significant.

Results

Stable uremic conditions, as defined above, were obtained in 80% of the animals that underwent renal resections and contralateral nephrectomies. The kidney function was normalized within a week and thereafter remained unaltered in the rats that underwent successful renal is transplantation.

The FST of the cardiac allograft in rats that had been uremic for 12 weeks was 12.2 ± 1.7 days. The duration of uremia did not affect the rejection times, thus no difference could be found between the studied uremia periods of 3, 6 and 12 weeks (table I).

After termination of the uremic state, heart allografts were rejected after 8.7 ± 0.4 days. In normal control animals, the FST was 8.6 ± 0.4 days and in the sham-operated rats, 8.4 ± 0.4 days. BN heart isografts all functioned for more than 60 days. The weight of the azotemic animals remained stable throughout the experiments and the serum albumin levels were unchanged (tables II, III). No significant alterations in serum hemoglobin levels were found. The number of WBC and lymphocytes remained unaltered without statistical differences between the various test groups.

Table I. Functional survival time (days) of heart allografts after different periods of uremia and after uremia reversal

	Duration of uremia			Reversed uremia	Controls BN rats	
	3 weeks (n = 5)	6 weeks (n = 5)	12 weeks (n = 10)	(n = 10)	normal (n = 10)	sham-operated (n = 10)
Mean $\pm$ SD, days	11.6 ± 1.4	11.7 ± 1.2	12.2 ± 1.7	8.7 ± 0.4	8.6 ± 0.4	8.4 ± 0.4
Range, days	11.0-14.0 ¹	11.0-13.5 ¹	9.5-14.5 ¹	8.0-9.0 ²	8.0-9.0	8.0-9.0

¹ p < 0.01 versus controls (Student's test). ² p < 0.01 versus values before uremia reversal.

Table II. Laboratory parameters and weights of uremic rats

	Time after induction of uremia			
	- 1 week	3 weeks	6 weeks	12 weeks
Weight, g				
Mean $\pm$ SD	236 ± 63	177 ± 12	212 ± 41	225 ± 50
Range	175-340	160-185	170-260	185-315
n	20	5	5	10
Serum creatinine, $\mu\text{mol/l}$				
Mean $\pm$ SD	58 ± 6	375 ± 54^a	349 ± 72^a	324 ± 84^a
Range	49-67	310-445	302-460	212-507
n	20	5	5	10
Serum albumin, g/l				
Mean $\pm$ SD	34 ± 1	31 ± 3	31 ± 2	30 ± 3
Range	32-35	30-37	29-34	27-35
n	5	5	5	10
Hemoglobin, g/l blood				
Mean $\pm$ SD	12.5 ± 1.1		9.2 ± 1.0^b	10.2 ± 2.9
Range	10.9-14.6		8.3-10.7	6.9-11.5
n	20		5	10
WBC/mm ³				
Mean $\pm$ SD	$11,600 \pm 4,920$	$9,900 \pm 2,100$	$8,900 \pm 1,640$	$6,790 \pm 4,160$
Range	7,400-14,000	6,400-11,300	6,900-10,600	1,700-12,200
n	5	5	5	10
Lymphocytes/mm ³ blood				
Mean $\pm$ SD	$6,400 \pm 1,180$	$4,360 \pm 910$	$3,320 \pm 750$	$5,790 \pm 1,170$
Range	3,020-13,200	7,180-1,430	1,940-6,200	900-10,790
n	10	6	5	10

^a p < 0.01 versus preoperative values. ^b p < 0.05 versus preoperative values.

Table III. Laboratory findings and weights before and after reversal of uremia

	Time after reversal of uremia		
	-1 day	7 days	14 days
Weight, g			
Mean $\pm$ SD	276 $\pm$ 13	272 $\pm$ 13	282 $\pm$ 12
Range	265-300	260-290	255-290
n	10	10	10
Serum creatinine, μ mol/l			
Mean $\pm$ SD	315 $\pm$ 155	68 $\pm$ 27 ^a	70 $\pm$ 18 ^a
Range	181-305	41-106	43-100
n	10	5	9
Hemoglobin, g/l blood			
Mean $\pm$ SD	9.6 $\pm$ 1.3	12.9 $\pm$ 0.6	10.8 $\pm$ 1.6
Range	7.6 $\pm$ 11.8	12.1-13.6	8.4-13.0
n	10	5	10
WBC/mm ³			
Mean $\pm$ SD	9,190 $\pm$ 1,030	10,960 $\pm$ 1,480	8,840 $\pm$ 1,980
Range	7,000-10,700	8,800-12,800	5,600-10,400
n	10	5	5
Lymphocytes/mm ³ blood			
Mean $\pm$ SD	5,840 $\pm$ 530	8,120 $\pm$ 830 ^b	
Range	3,410-8,230	5,480-14,080	
n	10	10	

^a p < 0.01 versus prereversal values.

^b p < 0.05 versus prereversal values.

Discussion

In spite of the generally held opinion that uremia is accompanied by a state of immunodeficiency, the proofs behind this assumption are far from unanimous (1,21,28,34). The authors who find an immunosuppressive effect in their test models present a wide range of explanations for possible mechanisms (5,8,17,27,30). A good capacity to reject transplants requires a normal function of the whole immune system and therefore it is of interest to study transplant rejection in the presence of uremia. However, so far mainly skin transplant experiments have been presented. Such studies provide limited knowledge of possible immunosuppressive

effects after organ transplantation as skin grafts immunize the recipient by lymphatic channels and not by the blood stream (36). Also, evaluation of rejection times after skin transplantations is difficult. The development of microsurgical techniques in organ transplantation now allows more extensive surgical procedures to be performed in small, genetically defined animals. The heterotopic heart transplant model then permits rapid and precise assessment of the FST. In the present study, stable uremia was induced surgically in isogeneic rats. The FSTs of Ag-B, different heart transplants were prolonged from 8.6 ± 0.4 days to 11.6 ± 1.4 days after 3 weeks' duration of uremia. No further prolongation of graft survival occurred in rats that had been uremic for longer periods of time. The reversal of uremia extinguished the immunodeficiency within a short time, thus cardiac allografting 1 week after kidney transplantation consistently revealed normal rejection times. Thus, as a normal immunocompetence was rapidly restored, a catabolic state or poor nutritive status are less likely explanations of the impaired immunological capacity in uremia. Also, albumin and hemoglobin levels in serum were unchanged during the experiments and the animals were not leukopenic to a significant degree. The number of lymphocytes in peripheral blood varied widely and no significant changes could be identified between different experimental groups. These observations are in agreement with Ormrod and Miller (24) and Souhami et al. (32) while Alevy et al. (1) found significant leukopenia and lymphopenia in uremic rats. The weights of the animals remained stable during the test periods, although normal rats would be expected to gain weight during the same time period.

So far only few experiments with uremic animals undergoing transplantations have been published. Smiddy et al. (31) used skin allografts in acutely uremic rabbits and found a moderate prolongation of graft survival. Chronic uremia of 6 months' duration has been shown to produce extended survival times of skin allografts

in rats (23). A chemically induced azotemia in 4 dogs prolonged kidney graft survival from 7 to 15-21 days (16). A slight effect on rat cardiac allograft survival by short-lasting uremia has also been described (32).

Clinically, skin graft survival in uremic patients has been reported to be prolonged as compared to healthy volunteers (6). Also in uremic patients both cellular and humoral immune responses have been found to be decreased (3-5,13,18,29,37). Other authors have found a normal in vitro cellular reactivity (12,30) and even an increased PHA stimulation responsiveness of lymphocytes from uremic patients has been reported (7,26). Humoral factors have been proposed as a cause of impaired immune response by some (10,12,20,22,30) but not all (11) authors.

Several observations suggest that inhibitory factors in uremic serum are responsible for the immunosuppressive effect in chronic renal failure (9,15,19,20,35). The rapid normalization of immune competence after isogeneic renal transplantation supports the hypothesis that the factors are easily excretable. The composition of 'post uremia' rat urine is now studied in our laboratory in search for soluble factors with immunosuppressive effects.

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THE IMMUNOSUPPRESSIVE EFFECT OF STREPTOZOTOCIN INDUCED
DIABETES IN RATS

Short title: P. Konrad et al: Immunosuppressive effects of
diabetes in rats.

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SUMMARY

Streptozotocin diabetic isogenous Brown-Norway (BN) rats received heterotopic Wistar-Furth (WF) x BN F₁ heart transplants. The functional graft survival time after different durations of diabetes and during insulin treated diabetes was recorded. Some diabetic rats were challenged with sheep red cells and their spleens were used in PHA stimulation- and plaque forming assays.

A prolongation in heart transplant survival not dependent on the duration of diabetes was found. The prolongation disappeared promptly when insulin was administered to the diabetic rats. A depression of cellular immuneresponsiveness as measured by PHA stimulation assay was recorded during diabetes. Valid results could not be obtained in the plaque forming assay.

KEY WORDS

Experimental diabetes, immunosuppression, rat, heart transplantation, streptozotocin.

It is widely accepted that individuals with insulin dependent diabetes mellitus have an impaired ability to respond immunologically (1). Few attempts have been made to quantitate this immunosuppressive effect in vivo, to determine whether insulin treatment results in a reversal, or to investigate whether the depression involves cellular or antibody mediated immunological events.

In the experiments reported here the effect of Streptozotocin induced diabetes on the survival times of heterotopic allogeneic transplants was investigated. Insulin was administered to some of the diabetic animals. The immune responsiveness was also investigated with in vitro assays.

MATERIALS AND METHODS

General outline of the experiments

Diabetes was induced in isogenous BN rats of both sexes. Heart transplantations were carried out at different time intervals using Ag-B different sex-matched isogenous rats as donors. One group of rats were treated with insulin prior to the grafting and until rejection occurred.

Other diabetic rats were challenged with sheep red blood cells and sacrificed for phytohaemagglutinin (PHA) and spleen cell plaque forming (Jerne) assay. Some of these diabetic rats had received insulin during 7 days before the tests.

Animals: Isogenous Brown Norway (BN) rats (150-290 g) and F₁ hybrids (170-250 g) from the BN and Wistar-Furth (WF) parental strains were used. They were fed on water and pellets ad libitum.

Induction of diabetes

A single intravenous dose of 50 mg/kg Streptozotocin (STZ) (U-9889, lot number 60, 273-2 from the Upjohn Company, Kalamazoo, Michigan) was injected into the tail vein of the rats under light

ether anaesthesia. The solution was prepared freshly in cold citratebuffer (pH 4.5). The rats that had serumglucose levels above 15 $\mu\text{mol/l}$ two days after injection were considered to be "diabetic". Approximately 90 % of the injected animals developed diabetes according to this definition. (Normal glucose levels were 4.8 - 6.2 $\mu\text{mol/l}$). The features of diabetes in rats could be observed after approximately one week. The animals started to lose weight and muscle mass and developed distended abdomens. Later cataracts occurred. Polydipsia, polyphagia and polyuria were apparent. No spontaneous regression of the diabetic state occurred (blood glucose range 18.5 - 28.0 $\mu\text{mol/l}$) but 25 % of the animals suffered from progressive disease and lost the urge for food. These animals were considered too sick for further experiments. They constantly died within 6 weeks after diabetes induction.

Experimental groups

(BN $\times$ WF) F₁ rats donated hearts to BN recipients.

- A. 5, 10 and 7 animals received heart transplants 4, 8 and 12 weeks respectively after diabetes induction.
- B. 5 rats received daily insulin treatment starting 7 weeks after diabetes induction. One week later the rats were allotransplanted.
- C. 19 BN rats were made diabetic by STZ injections. After 4 (11 rats) or 12 (6 rats) weeks of diabetes the animals were used in PHA stimulation assay. Two rats, diabetic since 8 weeks, had received insulin for one week at the time of the in vitro experiments. 20 rats were made diabetic in the same fashion for plaque forming assay experiments.
- D. 10 untreated control animals received cardiac allografts.

Heart transplantations

The procedures were performed under ether anesthesia with the use of the microsurgical technique described by Ono and Lindsay (2). Ischemic times were less than 30 minutes. The technical failure rate (postoperative mortality or non beating transplants after 3 days) was below 5 % in control animals. However, a considerably higher technical failure rate, (approximately 30 %), occurred in diabetic rats. The functional survival time (FST) was registered by palpation of the transplant in unanesthetized animals twice daily, thus allowing the determination of rejection times with 0.5 days' accuracy. When no heart beats were palpated an exploration of the graft was carried out to ascertain FST by macroscopical examination. In three cases the FST was then prolonged with 0.5 days.

Insulin treatment

"Very long acting Ultralente Novo" (40 IE/ml, Novo Industries, Denmark) was administered subcutaneously under aseptic conditions in a hind leg. In the rat this preparation is suitable for blood glucose control by one injection per day (3). During the first week of insulin treatment non-fasting capillary blood glucose levels were determined daily. 2 - 8 IU of insulin were given two hours after blood sampling. Later when blood glucose became stable, the levels were determined twice weekly. No insulin was given to animals with lower glucose levels than 4 $\mu\text{mol/l}$, as a single hypoglycaemic episode often turned out lethal in spite of glucose administration. No insulin was given on the day of surgery. It was possible to achieve blood glucose levels between 1,8 and 9,6 $\mu\text{mol/l}$ in 75 % of the animals and these were used in further experiments. The blood glucose decreasing effect of the insulin diminished after 3 weeks, probably because of development of anti bovine immunity.

Phytohaemagglutinin (PHA) assay

Rats were immunized by washed sheep red blood cells (1 ml of a 10 % suspension), injected i.v. 5 days prior to splenectomy. The spleens were divided into two halves and each half was mashed with a glass tube and the spleen cells were then suspended in RPMI 1640. After addition of iron particles the cells were incubated at 37°C for 45 minutes. Lymphocytes were isolated by centrifugation on FicollIsopaque as described by Bøyum (4). Triplicate-samples from each spleen half were cultured in microtitre plates containing 2.5×10^5 lymphocytes/culture in 200 μ l of RPMI 1640 containing 5% rat serum, glutamine 2 mM/ml, 2-mercapto ethanol 1:250.000 and gentamycin 12 μ g/ml. PHA (Wellcome) was added in final concentrations ranging from 5 to 1.25 μ g/ml.

The cultures were incubated for three days in 5 %CO₂ at 37°C and 22-24 hours before termination of cultures 0.5 μ Ci of ³H-thymidine (NET-027Z, specific activity 48 Ci/mM NEN, Boston, Mass.) in 50 μ l RPMI 1640 was added. Cultures were harvested onto glass fibre filters and rinsed in distilled water by use of a Skatron harvesting machine. The filters were dried and transferred to scintillation vials containing 4 ml of InstaFluor solution (Packard). The radioactivity was measured in a Packard Tri-Carb liquid scintillation counter. The median CPM value from each triplicate set of cell cultures was used in the statistical calculations. On each test occasion spleen cells from one or two diabetic and one normal rat were stimulated by PHA. Only the results from the tests where a satisfactory PHA stimulation had been obtained in the cells from the control rat were included in the results.

Plaque forming assay

The method described by Jerne (5, 6) was used. 20 diabetic and 8 control animals were tested.

Laboratory tests

Blood samples for determination of serum glucose and blood haemoglobin as well as total number of leucocytes and lymphocytes in blood were obtained repeatedly from the tail vein of unanesthetized animals. Serum samples for albumin and creatinine determinations were obtained, once from each rat, by direct puncture of the aorta at the time when the rat was sacrificed. Thus the creatinine and albumin values in table 2 and 3 were obtained from separate non transplanted rats. This was done to avoid a possible influence on the graft survival times by previous major blood sampling. Routine laboratory techniques of the hospital were used.

Statistical methods

P values $<0,01$ in Rank-sum test were considered "significant".

RESULTS

Non diabetic control rats rejected their allografts after 8.6 ± 0.4 days. The transplant functional survival times (FST) in diabetic animals that received heart allografts 4, 8 and 12 weeks after induction of diabetes are shown in table 1. FST was 18.1 ± 4.8 days after 4 weeks diabetes duration. No further prolongation of the transplant survival occurred in rats that had been diabetic for 8 and 12 weeks. "Non diabetic" rejection times were observed in the diabetic rats that had been treated with insulin for one week.

Table 1. Functional survival time (days) of heart allografts in diabetic and control rats.
Mean $\pm$ SD
(range)/n/

DURATION OF DIABETES				CONTROLS
4 weeks	8 weeks	12 weeks	8 weeks (1 w insulin treatment)	
$18.1 \pm 4.8^{***}$ (13.5-25.0)/5/	$18.6 \pm 4.5^{***}$ (13.0-25.5)/10/	$19.1 \pm 4.4^{***}$ (14.5-25.5)/7/	8.5 ± 0.8 (7.5-9.5)/5/	8.6 ± 0.4 (8.0-9.0)/10/

*** $p < 0.001$ versus non diabetic animals (controls)

Table 2. Laboratory tests and weights of diabetic rats. Mean $\pm$ SD (range) /n/.

Time after induction of diabetes (weeks)	0 (controls)	4	8	12
Albumine g/l	31 $\pm$ 2 (29-35)/7/	29 $\pm$ 3 (26-33)/7/	29 $\pm$ 2 (27-33)/8/	25 $\pm$ 3*** (21-30)/7/
Creatinine μ mol/l	53 $\pm$ 3 (50-59)/15/	56 $\pm$ 4 (51-60)/5/	58 $\pm$ 6 (49-75)/8/	66 $\pm$ 9** (58-82)/7/
Haemoglobine g/l	14.0 $\pm$ 2.3 (10.7-17.8)/12/	12.7 $\pm$ 1.5 (11.0-15.0)/9/	12.8 $\pm$ 1.7 (11.0-15.5)/7/	12.1 $\pm$ 2.7 (9.0-15.8)/7/
WBC ₃ /mm ³	13100 $\pm$ 2800 (8100-15000)/14/	11300 $\pm$ 2000 (8400-13000)/11/	9600 $\pm$ 2000 (7000-13200)/7/	7000 $\pm$ 1800*** (5400-10400)/7/
Lymphocytes /mm ³	10500 $\pm$ 1900 (7600-13000)/14/	7900 $\pm$ 2200 (3900-12300)/11/	5900 $\pm$ 1800*** (3500-8900)/8/	4100 $\pm$ 1600*** (2100-7000)/7/
Weights, % Of prediabetic value	100/30/	79 $\pm$ 5** (71-85)/10/	70 $\pm$ 6*** (60-81)/10/	79 $\pm$ 4** (73-86)/10/

*** p < 0.001; ** p < 0.01; * p < 0.05; versus control animals

Table 3. Laboratory findings and weights in diabetic rats treated with insulin. Mean $\pm$ SD (range)/n/.

Duration of diabetes (weeks)	0	8 (insulin treatment for 1 week)
Albumine g/l	31 $\pm$ 2 (29-35)/7/	32 $\pm$ 2 (28-33)/5/
Creatinine μ mol/l	53 $\pm$ 3 (50-59)/7/	52 $\pm$ 2 (50-55)/5/
Haemoglobin g/l	13.0 $\pm$ 1.6 (11.0-15.8)/7/	10.2 $\pm$ 1.0 (8.7-10.8)/5/**
WBC ₃ /mm ³	11300 $\pm$ 2000 (8800-14000)/5/	10800 $\pm$ 2500 (7600-15400)/5/
Lymphocytes /mm ³	8300 $\pm$ 2100 (6600-11300)/5/	7800 $\pm$ 2900 (5000-12200)/5/
Weights g	240 $\pm$ 44 (190-330)/10/	240 $\pm$ 40 (190-300)/10/

** p < 0.01 versus prediabetic values

Serum albumin and creatinine levels were unchanged after four and eight weeks of diabetes (Table 2). The albumin values were significantly lower after 12 weeks diabetes duration and a slight rise of serum creatinine could be observed after the same period. The haemoglobin levels in blood did not change during the diabetic periods. The number of lymphocytes were significantly lower after 8 and 12 weeks of diabetes. After 7 weeks of untreated diabetes and one week of insulin treatment albumin and creatinine values were not significantly different from prediabetic ones (Table 3). The haemoglobin levels were decreased.

Creatinine and albumin levels in serum were also determined after rejection of a heart allograft when the animals were sacrificed. The values did not differ significantly from the ones recorded in non-transplanted rats that had been diabetic for a similar time period. The body weight decreased progressively in diabetic animals but returned rapidly to the prediabetic levels during insulin treatment.

The ^3H -thymidine uptake by spleen cells treated with PHA was reduced after four and twelve weeks of diabetes (Table 4). Approximately 40 % of the in vitro experiments had to be discarded due to inadequate stimulation of the control animal spleen cells. In the plaque forming assay experiments the number of plaques recorded in control BN rats were too variable to allow meaningful analyses.

Table 4.

Median ^3H Thymidin uptake by spleen cells after treatment with PHA; cpm, (range)/n/

Duration of diabetes (weeks)	4	12	8 (1 w insulin treatment)
Diabetic animals	17500 ** (5200-26500)/11/	6700 ** (2600-39100)/6/	66300 ¹⁾ 64300
Control animals	39200 (17600-59400)/13/		

1) two experiments

** $p < 0.01$ versus control animals

DISCUSSION

A normal capacity to reject allogeneic organ transplants requires an immune response system that functions satisfactorily in all its branches and all consecutive steps. Therefore an allogeneic organ transplant model within two isogenous strains with exact and standardized rejection times gives information whether an immunosuppressed state is present or not. The strength of the effect can be determined and compared with that of other immunosuppressive states. For example the prolongation in transplant survival recorded in our diabetic rats was more pronounced than that obtained in the same model under uremic conditions (7). Analysis of the mechanisms behind a disclosed effect requires the use of in vitro assays.

In the experiments reported here prolonged functional survival times of allogeneic heart transplants were found in rats that had been diabetic during four weeks. The effect was not augmented by long diabetes duration and was abolished a short time after institution of insulin treatment. The animals had normal blood haemoglobin, serum creatinine and serum albumin levels when an in vivo immunosuppressive effect was recorded after four and eight weeks of diabetes. The body weights were reduced. Also as the diabetic rats constantly had large amounts of bowel contents at autopsy a more pronounced "true" weight loss could be masked. After eight weeks of diabetes including one week of insulin treatment the in vivo immunosuppressive state had disappeared, while serum albumin levels were still normal and body weights had been normalized. The slight anemia noted in the insulin treated rats was probably caused by the frequent blood sampling for blood glucose determinations. Thus, 1) the immunosuppressive effect was completely reversed as soon as heart transplantations could technically be carried out in normoglycemia, and 2) the magnitude of transplant survival prolongation was not influenced by the duration of diabetes. It is therefore not likely that the immunosuppressive effect recorded in non insulin treated diabetic animal was a pure con-

sequence of catabolism. The compromised immune response sequences must be insulin dependent as the insulin treated, but still Streptozotocin-diabetic animals, had an intact capacity to reject allogeneic organ transplants.

Attempts were made to analyse the mechanisms behind the immunosuppressive effects by use of in vitro assays monitoring cell mediated (PHA) and antibody-mediated (plaque forming assay) immune responses. Spleen cells in diabetic rats responded weaker to PHA stimulation than those from normal rats. When insulin had been given the stimulation by PHA was not decreased on two occasions. The results suggest that a diminished cell-mediated immune response is one of the factors causing immunosuppression in streptozotocin (STZ) diabetic rats. Unfortunately, the plaque forming assay for detecting possible antibody-mediated immune response did not give reproducible results. Brown Norway spleen cells might have an unfortunate low reactivity in in-vitro tests as in both assays valid results were constantly obtained in the same laboratory with spleens from other rat strains.

The immune deficiency of experimental STZ induced diabetes has previously been studied in rats and mice. Cell-mediated immune responses are primarily affected (8, 9) but decreased antibody production and deficient macrophage function has also been reported (10, 11). Transplant experiments in diabetic animals have only been performed with skin grafts known to immunize by a lymphatic route instead of the intravenous way of organ grafts. The survival of skin grafts has been reported to be prolonged (8). Some authors have found normal rejection times of first set grafts but prolonged survival of second set ones (12). Insulin treatment has generally been found to, at least partly, reverse the immune deficiency (10, 11,). A reversal as measured by in vitro assays has also been demonstrated with suboptimal non-normoglycemia inducing, doses of insulin (8, 12). In contrast Nichols et al (13)

found unchanged growth of syngenic tumors in diabetic mice during insulin treatment.

STZ is an alkylating agent whose diabetogenic effect is thought to be mediated by a reduction of Nicotinamideadenine-dinucleotide in pancreatic β cells (14). It might also induce diabetes by an autoimmune mechanism mediated by T cells responding to antigens released from pancreatic β cells, damaged by a direct (minor?) cell toxic effect by STZ. Several data are in favour of such an hypothesis, for instance nude mice with deficient T-cell function are resistant to the diabetogenic effect of STZ (15). The toxic effect might also act upon lymphoid cells. Bone marrow cells from STZ diabetic mice are less effective than normal ones to protect lethally irradiated syngenic non diabetic mice from death after bone marrow cell transfer (9, 16). The direct toxicity by STZ on mice lymphoid cells as measured by MLC reactivity has been found to reverse spontaneously after 20 days, that is before the time of organ transplantation in our experiments (9). Furthermore, in the present experiments insulin treatment rapidly restored a full capacity to reject transplants. It is therefore not likely that the immune deficiency in our STZ induced diabetic rats was caused by a direct toxic effect on lymphoid cells.

Clinical studies on the immune competence of diabetic patients are somewhat contradictory. The response to PHA stimulation has been found to be impaired, more in insulin treated than in noninsulin dependent diabetic patients (17). On the other hand Brody and Merlie (18) found a depression in PHA responsiveness particularly in hyperglycemic diabetic patients and suggested a metabolic disturbance rather than an immunological abnormality. Also cell mediated immune responses as measured by lymphocyte transformation experiments have been found to be normal in well controlled insulin-dependent diabetics (19). Suppressor cell activity is significantly reduced in newly discovered insulindependent individuals as

compared to patients with long standing diabetes disease and healthy persons (20).

Should one dare to draw parallels between "mice and men" the results presented here suggest that the optimally insulin treated diabetic organ transplant patients are not in an immune deficiency state in addition to that created by the immunosuppressive treatment.

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ENHANCED GROWTH OF COLON CARCINOMA ISOGRAFTS IN RATS CON- CURRENTLY RECEIVING ORGAN ALLOGRAFTS

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Patients with organ transplants or immune deficiency diseases have an increased incidence of malignant disorders (1). Immunosuppressive drugs are known to cause such an increased incidence and have been implicated as the cause of the posttransplantation malignancies (2).

In the experiments reported here another possibility is tested. A strong antigenic stimulus from an organ allograft may occupy the immune defense system to the extent that the cancer homeostasis processes are weakened.

MATERIALS AND METHODS

Groups of isogenous Brown-Norway (BN) rats received BN/WF (Wistar-Furth) F₁ and syngeneic BN heart transplants, respectively. At different time intervals, a standardized dose of syngeneic BN tumor cells were injected subcutaneously into one hind leg of each organ graft recipient. The tumor growth rates in the two groups

receiving organ allo-and isografts were compared.

Colonies of isogenous BN and WF rats were maintained on an ad lib water and pellet diet (Astra-Ewos rat-pellets R3). Male or female rats (200-250 g) were used as transplant recipients. All cardiac grafts were derived from female animals.

Heterotopic heart transplantations were performed under barbiturate anesthesia using the methods of Ono and Lindsay (3). Transplant function was assessed daily by palpation. The allotransplanted hearts ceased to function 8.5 days (mean) after the surgical procedures, whereas 100% of the isografts pulsed throughout the observation period.

DMH-BN 12 tumor cells were used. This colonic carcinoma was originally induced with dimethylhydrazine (**DMH**) in a **BN** female and used as the 14th to 22nd serial passages in **BN** rats. Two-hundred microliters of a standardized cell suspension were used for each subcutaneous hind leg inoculation. Aliquots of the same tumor cell suspension were inoculated simultaneously to both the test and the control group animals. Tumor size was measured weekly by caliper for 8 weeks, and tumor volume was calculated by a method described by Attia and Weiss (4). The difference in tumor cell volumes between groups receiving organ allo- and isografts, respectively, was evaluated for statistical significance using the Mann-Whitney U test.

RESULTS

A total of 83 isogenic and allogeneic heart transplantations were performed. The technical failure rate (death or nonbeating transplant within 1 week after the transplantation) was 9.6%. Rats receiving iso- or allotransplants 2 or 6 days after tumor challenge showed no statistical difference in tumor growth rate. A significant ($p < 0.02$) increase in tumor size was observed after 4 weeks in

the allotransplanted rats as compared to the isografts, when heart transplantation was performed 2-4 days before the tumor cell inoculation (Table 1).

In a separate pilot study, the tumor sizes in 12 rats receiving allo- or isografts while receiving cyclophosphamide treatment (10 mg/kg/day, intramuscularly) were compared. The tumor growth rates were depressed to a variable extent, and no significant difference in tumor volumes could be found.

Table 1. Tumor Cell Volumes (Mean $\pm$ SD) at Different Intervals After Tumor Cell Challenge (cu mm)

	Weeks After Tumor Cell Challenge			
	2	3	4	5
Allotransplant 2 - 4 days before tumor cell challenge (13 rats)	92 $\pm$ 40		244 $\pm$ 166	388 $\pm$ 201
Isotransplant 2 - 4 days before tumor cell challenge (15 rats)	60 $\pm$ 32		121 $\pm$ 98	287 $\pm$ 202
p Value*	NS		p < 0.02	NS
Allotransplant 2 - 6 days after tumor cell challenge (11 rats)	46 $\pm$ 41	69 $\pm$ 56	250 $\pm$ 195	404 $\pm$ 366
Isotransplant 2 - 6 days after tumor cell challenge (13 rats)	38 $\pm$ 34	58 $\pm$ 38	187 $\pm$ 146	272 $\pm$ 183
p Value*	NS	NS	NS	NS

*Statistical significance of differences in tumor volume between corresponding allo- and isotransplanted test groups evaluated by the Mann-Whitney U test. NS, not significant.

DISCUSSION

Although the well documented increased frequency of malignant disorders associated with organ transplantation is usually regarded as a side effect of the immunosuppressive treatment, alternative mechanisms are at hand. Thus, the relationship between allogeneic transplantation and malignancies can also be viewed in the context of antigenic competition or nonspecific antigen-induced suppression of the immune response (5, 6).

When strong and weak antigens are administered together, antigenic competition may cause an individual to respond only to the stronger antigen. It is considered to be an important factor in viral oncogenesis, but could also be of importance when histoincompatibility and nonviral tumor-associated antigens are administered simultaneously. Antigenic competition can cause suppression of both IgM and IgG antibody formation, delayed hypersensitivity, graft-versus-host (GVH) reactions, graft rejection, and may suppress the induction of experimental autoimmune lesions (6). Chronic antigen stimulation, as for instance during a slow GVH reaction, or multiple experimental antigen injections can also cause an increased frequency of neoplasms.

In some experimental murine tumor systems, antigenic stimulation combined with immunosuppression can cause an increased tumor incidence (5). The relevance of these views to the field of transplantation has been supported by a previous demonstration that an experimental fast-growing polyoma virus-induced sarcoma (PW-13) grew significantly faster in rats carrying skin allografts (7). However, the kinetics of the immunization process resulting from a skin transplant are quite different compared to those from a directly vascularized transplant. Therefore, the results might not be relevant for an organ transplantation situation.

The present results demonstrating that an experimental tumor grew

significantly faster in nonimmunosuppressed recipients that also received histoincompatible organ transplants than in animals receiving syngeneic organ transplants confirm the validity of the antigenic competition concept in relation to the increased incidence of malignancy associated with organ transplantation.

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PROLONGED SURVIVAL OF HEART ALLOGRAFTS IN RATS TREATED WITH
ANTISERA TO β_2 -MICROGLOBULIN¹

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β_2 -microglobulin has been the subject of numerous recent studies because of its homology with immunoglobulin domains, its association with histocompatibility antigens, and its possible association with lymphocyte receptor structures (7, 13). Antibodies against β_2 -microglobulin have been shown to inhibit several lymphocyte proliferative responses, such as the mixed lymphocyte culture reaction (1, 10, 11, 17), the stimulation with phytohemagglutinin (1, 16), and the stimulation with the soluble antigen-purified protein derivative of tuberculin (1, 11, 17). These reactions are known to be carried out primarily by thymus-derived (T) lymphocytes. According to one investigation (2), anti β_2 -microglobulin also seems to inhibit the T lymphocyte effector function determined by the cell-mediated lympholysis assay.

¹ This work was supported by grants from the Swedish Medical Research Council and the Medical Faculty, University of Lund, Sweden. Because antisera against β_2 -microglobulin repress various T lymphocyte reactions in vitro, it seemed to be of interest to examine whether such antisera also can delay the

allograft rejection process which is largely effected by T lymphocytes.

We have studied the effect of treatment with anti- β_2 -microglobulin sera on the survival of heart allografts transplanted between two isogenous inbred rat strains. Hearts from male Wistar-Furth (WF) rats (Ag-B2) were grafted heterotopically into the peritoneal cavity of male 250- to 300-g animals of the Brown Norway (BN) strain (Ag-B3). The surgical technique of Ono and Lindsay was used (14). Graft survival was determined by daily palpations and, if necessary, by laparotomy. Isografts survived for several months (upper limit not determined). Two technical failures (nonbeating heart 24 hr after surgery) were recorded.

A goat anti-rabbit β_2 -microglobulin serum (4) was used in the first series of experiments. This antiserum was found to be cytotoxic not only to rabbit lymphocytes, but also to rat lymphocytes (L. Björck, B. Löw and I. Berggård, unpublished data) and to precipitate purified rat β_2 -microglobulin on immunodiffusion analyses (L. Lögdberg, P.O. Östergren and I. Berggård, unpublished data). A second series of experiments was performed with a rabbit antiserum (5) against rat β_2 -microglobulin. In a two-step lymphocytotoxicity test (18), the cytotoxic titres of the two antisera (dilutions inducing 50% lysis of BN lymphocytes) were 1:8 (goat anti-rabbit β_2 -microglobulin serum) and 1:16 (rabbit anti-rat β_2 -microglobulin serum). Both antisera showed only one precipitin arc when tested on immunoelectrophoresis against varying amounts of concentrated urinary rabbit or rat proteins.

Control groups of rats received pooled nonimmune goat serum, pooled nonimmune rabbit serum, or rabbit anti-human albumin serum. All three control sera were noncytotoxic to BN lymphocytes. The rabbit anti- β_2 -microglobulin and antialbumin sera were obtained by the same immunization procedure (5). The anti-BN erythrocyte

agglutination titres were 1:4, 1:8, and 1:16 in all three rabbit sera, the goat anti-rabbit β_2 -microglobulin serum, and the pooled nonimmune goat serum, respectively.

All sera were heat-inactivated at 56 C for 30 min, dialyzed against phosphate-buffered saline, pH 7.4, and passed through Swinnex-25 filters (Millipore). One-ml portions of sera were given i.v. as indicated in Table 1.

Table 1. Survival of heart transplants after treatment with anti- β_2 -microglobulin sera, rabbit anti-human albumin serum, and nonimmune sera^a

Goat anti-rabbit β_2 -microglobulin serum	Graft survival, days for each serum-treated rat				Rabbit anti-human albumin serum
	Pooled nonimmune goat serum	Rabbit anti-rat β_2 -microglobulin serum	Pooled nonimmune rabbit serum		
8	7	8	7		7 ^b
9	6	9	7		7 ^b
8	7	8	7		
8	7	10 ^c	7 ^b		
10 ^c	7 ^b	8 ^c	7 ^b		
Mean $\pm$ SD 8.6 $\pm$ 0.8	6.8 $\pm$ 0.2	8.6 $\pm$ 0.8	7 $\pm$ 0		7 $\pm$ 0

^aExcept where indicated the recipient was given 1 ml of serum on the day before operation, 1 ml at the time of transplantation (day 0), and 1 ml on days 1 to 6 (total dose = 8 ml).

^bTreatment was proceeded until rejection (total dose = 9 ml).

^cTreatment was proceeded for another 2 days (total dose = 10 ml).

The transplant survival times are summarized in Table 1. Twenty-five rats were grafted without serum treatment. The functional graft survival time was 6.84 ± 0.14 days (mean $\pm$ SD). The survival of the allografts in five animals that received goat antirabbit β_2 -microglobulin and in five animals that received rabbit anti-rat β_2 -microglobulin was in both instances 8.60 ± 0.80 days. The difference in graft survival between groups that were given

anti- β_2 -microglobulin sera and nonimmune sera was statistically analysed by the Mann-Whitney U test. The survival time after anti- β_2 -microglobulin serum administration was significantly prolonged ($P < 0.01$).

The prolongation of allograft survival is comparatively small (8). One possible explanation for this could be absorption of antibodies by β_2 -microglobulin present on the recipients nonlymphoid tissues, thereby leaving only a small amount of anti- β_2 -microglobulin to procure allograft survival.

In several studies permanent graft survival has been induced by enhancing alloantisera or antilymphocyte sera in certain rat strain combinations. In these experiments, however, semiallogeneic (F_1) donors have usually been used. The survival times have been considerably reduced when the gene dose differences between donors and recipients have been increased (8).

The WF-BN histocompatibility barrier is strong (3). This was also observed by grafting hearts from WF rats into six BN animals treated with a rabbit anti-BN lymphocyte serum (9) in a similar protocol. The prolongation of transplant survival time was 2 to 4 days (P. Konrad and B. Husberg, unpublished data). This is only slightly more than the prolongation caused by anti- β_2 -microglobulin. It should be possible to achieve longer allograft survival times with anti- β_2 -microglobulin by the use of weaker histocompatibility barriers. However, the purpose of this study was more to establish that anti- β_2 -microglobulin has graft protective properties than to determine the strength of the immunosuppressive effect.

As described above, all sera used in this study had low titres of agglutinins against BN rat erythrocytes. These agglutinins do not seem to influence the survival of the allografts, since the time

of rejection was the same in untreated rats and in animals treated with nonimmune or antihuman albumin sera.

Antisera against human β_2 -microglobulin have been reported to be mitogenic to bone marrow-derived (B) lymphocytes in vitro (12, 15, 16). Such effects can hardly explain the prolonged allograft survival observed in this study.

T lymphocyte-mediated immune reactions are regarded as the most important in the rejection of first-set transplants. Antibodies to β_2 -microglobulin could interfere with the rejection process by reacting with responding T lymphocytes, with the allograft or with both.

It seems likely that the prolonged allograft survival observed in this study is at least partly attributable to interference with T lymphocyte functions. As mentioned above, it has been shown that antibodies against β_2 -microglobulin inhibit the mixed lymphocyte culture (1, 10, 11, 17) and cell-mediated lympholysis (2) reactions and also the antigenic stimulation of lymphocytes with purified protein derivative (1, 11, 17). Studies from two laboratories indicate that anti- β_2 -microglobulin inhibits the responding cells in the mixed lymphocyte culture reaction (10, 11). The combined evidence from all of these experiments suggests that β_2 -microglobulin is part of, or associated with, receptor structures on T lymphocytes. Anti- β_2 -microglobulin could act by blocking or modulation of such receptor structures and by complement-dependent cytotoxicity. The possible contribution of cytotoxicity can be explored by treatment of allograft recipients with $F(ab)_2$ fragments of anti- β_2 -microglobulin.

It is an established fact that β_2 -microglobulin is part of the major serologically defined transplantation antigens (7, 13). Thus, anti- β_2 -microglobulin might also act as enhancing anti-

bodies by binding to such antigens in the transplant. The nature of enhancing antibodies in the rat model is, however, not clear. According to a recently published report, the predominant part of such antibodies do not have specificity to the Ag-B antigens, but rather to antigenic structures that may represent Ia specificities (6). β_2 -microglobulin apparently is not associated with Ia antigens (19).

In summary, antibodies against β_2 -microglobulin have been shown to cause a significant but moderate prolongation of the survival of rat heart allografts. It is suggested that the prolongation is at least partly attributable to interference with T lymphocyte functions.

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PROLONGATION OF HETEROTOPIC RAT HEART ALLOGRAFT SURVIVAL BY
"METHOTREXATE-FOLINIC ACID RESCUE"

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Key words. Immunosuppressive agents, Methotrexate, Organ transplantation

ABSTRACT. Brown Norway rats received heterotopic (WF/BN) $\times$ F_1 heart transplants. Functional graft survival time (FST) was 10.0 ± 0.5 days. When the highest tolerable daily dose of methotrexate (25 μ g/kg) was given, FST was 26.2 ± 5.6 days. When the highest tolerable dose of methotrexate with "folinic acid rescue" (175 μ g/kg) was administered, the FST was found to be 25.3 ± 11.0 days. Folinic acid given between daily methotrexate doses diminished the toxicity of the latter drug but also its therapeutic transplant-protective effectiveness. Methotrexate alone given in multiple nontoxic doses proved to be a strong and safe transplant-protective immunosuppressive agent.

Methotrexate, a folic acid antagonist, has a well-known immunosuppressive capacity but its usefulness has been hampered by a reported considerable toxicity (9, 16). Attempts have been made to diminish the side-effects by administration of an antidote, folinic acid, intermittently between the methotrexate injections. This "folinic acid rescue" concept has been claimed to lessen side-effects without marked loss of therapeutic effects in tumor treatment (14) and after skin transplants (1, 4). However, experi-

ments using organ transplantation models have not given any clear evidence whether methotrexate (with or without "folinic acid rescue") is a useful posttransplantation immunosuppressive agent (7, 8, 11, 13, 16).

In this study the effectiveness of the folinic acid rescue principle was examined using a highly standardized allogenic cardiac transplantation model in rats. The effect of methotrexate alone and different methotrexate-folinic acid combinations were studied.

Material and Methods

General Outline of the Experiments

Two isogenous rat strains, Brown Norway (BN) and Wistar Furth (WF), were used. BN rats were given heterotopic (BN/WF) $\times F_1$ rat heart transplants. Functional survival time of the grafts were recorded after the use of different methotrexate-folinic acid administration protocols.

Drug Administration

All drugs were given intravenously through a polyethylene catheter introduced into the inferior caval vein through the right femoral vein. The catheters ended distally in a modified venflon (Viggo AB^R) placed subcutaneously between the scapulas. A rubber membrane equipped side tube protruded through the skin. The catheter was rinsed with heparinized saline (3 IU in 0.3 ml) after each drug administration. The methotrexate doses were adjusted every other day according to weight loss of the animals.

Surgical Techniques

The heterotopic rat heart transplant model of Ono and Lindsay (12) was used. Male or female rats weighing 200-250 were used. The technical failure rate (nonbeating transplant 3 days postopera-

tively) was below 5%. 3 animals that experienced early (less than 7 days) sudden death with beating transplanted hearts were likewise excluded. Functional graft survival time (FST) was recorded by palpation and by laparotomy after cessation of palpable beats (2). Also, at this time the position of the infusion catheter tip was checked.

Experimental Groups

Toxicity Test (table 1). 23 BN rats underwent sham laparotomies and were given different doses of methotrexate (Lederle) intravenously every 24th h. In 14 rats the methotrexate injections were followed by 500 µg/kg of folinic acid (Leucovorin, Lederle) 16 and 20 h after each methotrexate injection.

Transplanted Test Groups (table 2). In one group of 24 animals different methotrexate doses were given daily until graft rejection. In a second group (17 rats) each dose was followed by injections of folinic acid, 500 µg/kg, 16 and 20 h later. The first dose of methotrexate was given within 1 h after graft vascularization.

Results

The results of the toxicity tests are shown in table 1. Surviving rats were treated during 3 weeks and were observed for further 2 weeks. Methotrexate, given in daily doses above 50 µg/kg, was found to be lethal. If 500 µg/kg of folinic acid was administered 16 and 20 h after each methotrexate injection, 175 µg/kg of methotrexate could be given daily without mortality. The rats that died during the toxicity tests showed a profound premortal leukopenia and weight loss.

Table 1. Toxicity of the different methotrexate and folinic acid doses

Methotrexate ug/kg/day	Folinic acid, $\mu\text{g/kg/day}$	
	0	500 x 2
10	2/2	-
25	3/3	-
50	1/4	2/2
100	0/2	-
125	0/4	4/4
175	0/4	4/4
200	0/4	1/4

Number of surviving/total number of treated rats.

The unmodified FST (BN/WF) x F₁ BN was found to be 10.0 ± 0.5 days (mean $\pm$ SD; table II). If the maximum tolerable doses of methotrexate with "folinic acid rescue" was administered, the FST was found to be 25.3 ± 11.0 days. When the highest tolerable methotrexate dose without folinic acid administration (25 $\mu\text{g/kg}$) was given, the graft survival time was 26.2 ± 5.6 days.

In the group of rats where a low (well tolerable), daily dose of methotrexate (10 $\mu\text{g/kg}$) was used alone, the graft survival was 19.2 ± 1.9 days. When folinic acid was added this time was reduced to 9.2 ± 2.2 days.

Table 2. Functional survival time of allogenic heterotopic heart transplant in days (mean $\pm$ SD)

Methotrexate $\mu\text{g/kg/day}$	Folinic acid, $\mu\text{g/kg/day}$	
	0	500 x 2
0	10.0 $\pm$ 0.5 (10)[10.0]	10;9
10	19.2 $\pm$ 1.9 (5) [20.0]	9.6 $\pm$ 0.9 (5)[9.0]
25	26.2 $\pm$ 5.6 (9)[23.0]	-
175	-	25.3 $\pm$ 11.0 (10)[23.5]

Median values are given within brackets; number of rats are given in parantheses.

All transplanted animals were in good condition at the time of graft rejection.

Discussion

Methotrexate is an inhibitor of the enzyme dihydrofolate reductase preventing the conversion of folic acid to tetrahydrofolic acid (6). This step is necessary for the synthesis of many biochemical compounds including DNA, RNA and several coenzyme species. It has a well documented and powerful immunosuppressive effect but also considerable toxic side-effects (3, 10, 11). Methotrexate has been reported to have a triphasic plasma disappearance rate with half-lives of 0.75, 3.5 and 27 h, respectively, after intravenous injection in man (17).

Folinic acid is a metabolite beyond the block and thus constitutes an antidote to methotrexate. The presence of folinic acid during

the long terminal half-life period is supposed to diminish the toxicity of methotrexate more than its therapeutic efficacy. Such folinic acid rescue protocols are usually based on empirical data and pilot experiments as there is insufficient knowledge of the disappearance rate of a given dose of methotrexate or folinic acid from the extracellular and intracellular compartments (15).

Folinic acid rescue has been used in the treatment of malignancies (14) and to achieve immunosuppression (10). The latter protocols have mainly reported on suppression of the immunological responses to pure antigens (5) or allogenic skin transplants (1). To our knowledge the folinic acid rescue principle has not successfully been used after organ transplantation (11).

In this study the "folinic acid rescue" principle after experimental organ transplantation was examined. Administration of folinic acid between daily methotrexate doses diminished the toxicity of the latter drug but also to the same degree its therapeutic, transplant-protective effectiveness.

So far only one time interval between each methotrexate and folinic acid dose has been tried. Further systematic studies on the effect on the graft protection capacity and toxicity by variations in methotrexate-folinic acid time intervals are now under way in our laboratory. Perhaps it will be possible to detect a time interval between methotrexate and folinic acid doses where the "folinic acid rescue" concept can be verified.

Nevertheless, the availability of a specific antidote is of great advantage, should a profound bone marrow depression or serious infectious complication occur in clinical use of methotrexate.

It is remarkable that methotrexate alone given in multiple, low, nontoxic doses proved to be a not only strong but also safe

transplant-protective immunosuppressive agent. The prolongation of functional survival time was marked in spite of the strong Ag-B different antigen barrier that was used in the experiments.

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THE EFFECT OF METHYLPREDNISOLONE ON HETEROTOPIC HEART TRANSPLANT SURVIVAL

Glucocorticoid agents have been a cornerstone in clinical post-transplantation immunosuppression for more than two decades. They have a well documented and powerful immunosuppressive effect. Therefore it is of interest to compare their influence on graft survival in the same system as that used in study I, II, IV and V.

MATERIALS AND METHODS:

General design of the experiments: Two isogenous rat strains, Brown Norway (BN) and Wistar Furth (WF) were used. BN rats were given heterotopic (BN x WF) F₁ rat heart transplant. Functional survival time (FST) of the grafts were recorded.

Experimental groups: Three groups of 5 rats were given methylprednisolone (Solumedrone^R) intravenously, 5 mg/kg twice a day, 20 mg/kg once a day and 20 mg/kg twice a day. A fourth group of 3 rats were given the drug in a dose of 40 mg/kg once a day. Seven untreated control BN rats received (BN x WF) F₁ heart transplants.

Surgical techniques: The technique described on page 9 was used. The steroid treated rats received the permanent catheter for repeated intravenous injections as described on page 14.

Statistical methods: A Rank-Sum test was applied.

RESULTS:

The functional transplant survival in non-immunosuppressed rats was 8.5 ± 0.4 days. (Fig 9). Five mg/kg of Prednisolone injected intravenously twice a day resulted in a marked prolongation in FST without the loss of animals or deteriorations in general condition. A dose of 20 mg/kg twice a day gave a superior graft

survival but two late deaths were obtained. Methylprednisolone administration once a day to allotransplanted rats resulted in a prolongation in graft survival only when toxic doses were given (40 mg/kg). Weight loss and cachexia was noted after long term treatment with daily methylprednisolone doses of 40 mg/kg.

Table I.

Survival of heterotopic (BN x WF) F₁ hearts in BN rats, days (Mean $\pm$ SD)

Daily steroid dose (mg/kg)	5 mg x 2	20 mg x 1	20 mg x 2	40 mg x 1	0(Controls)
Number of animals	5	5	5	3	7
Functional transplant survival time (days)	15.0 $\pm$ 1.7**	8.0 $\pm$ 0.4	69.4 $\pm$ 11.6 ††	20 [†] 25 [†] 20 [†]	8.5 $\pm$ 0.4

† Dead with functioning transplant.

†† Two rats dead with functioning transplants after 55 and 67 days respectively.

** p < 0.01 versus controls

